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JCMR Office: Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran.
Postal Code: 9177948953
P.O. Box: 917751436
Tel: +98-513-8804063
Fax: +98-513-8795162
E-mail: jcmr@um.ac.ir
Online Submission: http://jcmr.fum.ac

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Research Article

Association of Codon 72 of *P53* Gene Polymorphism with Chronic Hepatitis C Virus Infection: A Case Control Study

Sina Gerayli[1], Alireza Pasdar[2,3], Sina Rostami[4], Samaneh Sepahi[5], Seyed Mousalreza Hoseini[6], Mitra Ahadi[6], Reza Jahanian[7], Aida Gholoobi[2], Zahra Meshkat[7*]

[1] *Department of Biology, Western University, London, Ontario, N6A 5B7, Canada*
[2] *Department of Modern Sciences and Technologies, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[3] *Division of Applied Medicine, Medical School, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK*
[4] *The Influenza Centre, Department of Clinical Science, University of Bergen, N-5021Bergen, Norway*
[5] *Targeted Drug Delivery Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*
[6] *Department of Gastroenterology and Hepatology, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[7] *Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

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Abstract

Single nucleotide polymorphism in codon 72 of *p53* gene (Arg/Pro) changes *p53* protein structure and affects its activities. Hepatitis C virus (HCV) is believed to induce hepatocellular carcinoma and *P53* polymorphisms have been associated with human cancers. The aim of this study was to evaluate genetic variants of codon 72 of *p53* gene polymorphism in HCV patients and its relationship with HCV infection. The study was conducted on 67 HCV patients, who were referred to medical centers of Mashhad city, Iran, and 73 healthy people from the same region. Genotyping of codon 72 of *p53* gene was performed by PCR-RFLP method. The distributions of different alleles of *p53* polymorphisms did not differ significantly between groups. The respective proportions of Proline homozygotes, heterozygotes, and Arginine homozygotes were 37.31%, 35.82%, 26.86% in patients and 39.72%, 27.39%, and 32.87% in the control group respectively. However, we found no significant differenece for the allelic or genotype distribution between cases and controls. Our results indicated no strong evidence of association of the *p53* polymorphism with HCV infection; however, further investigation is needed in different ethnic groups to elucidate the role of this polymorphism in HCV infection.

***Keywords*:** Polymorphism, P*53* gene, HCV, Genetic epidemiology, Iran

Introduction

The effects of genetic and environmental factors have been definitively proved in cancers (Ziech et al., 2010a; Ziech et al., 2010b; Ziech et al., 2011). Genetic factors are not only effective in hereditary cancers such as bilateral retinoblastoma or xenoderma pigmentosum, but it seems that these factors play a significant role in common cancers and so far many related genes have been identified (Kraemer et al., 1987; Sepahi et al., 2014).

P53 mutations have extensively been studied in human tumours (Chen et al., 2010; Goh et al., 2011). *P53* has 11 exons and codes for a protein containing 393 amino acids (Marcel et al., 2011). *P53* has different functions such as DNA binding (Hagn et al., 2010) cell cycle control (Leontieva et al., 2010), DNA restoration (Sotiropoulou et al., 2010), differentiation (Molchadsky et al., 2010), genomic plasticity (Zhao and Xu, 2010), and programmed cell death (apoptosis) (Li et al., 2012). Therefore, the overall function of p53 is to maintain genomic integrity. Genetic polymorphisms are the natural differences of DNA nucleotide sequence which can cause variations in genes products or responses to different stimuli. Mutation of *p53* has a close relationship with cancers in patients who have Li-Fraumeni syndrome, which is caused by deactivation of preventive gene of *p53* tumour through mutation (Srivastava et al., 1990). Genetic polymorphisms of *p53* can be found in different locations of the gene and their relations with various malignancies have been underlined (Själander et al., 1995; Sjalander et al., 1996). In cervical cancers, *p53* Arg (CGC) at codon 72 has been associated with the disease in patients infected by HPV (Storey et al., 1998; Zehbe

Corresponding authors E-mail:
* meshkatz@mums.ac.ir

et al., 1999). However, many researchers have challenged this claim (Minaguchi et al., 1998).
Japanese researchers found that the frequency of *p53* Pro in patients who suffer from hepatocellular carcinoma is higher than non-cancerous cases (Xiong et al., 2009). Most of these patients were infected with hepatitis C virus (HCV).
We aimed to assess if polymorphism in this gene could increase the risk of HCV infection in our population. Accordingly, in the present study, *p53* Arg72Pro single-nucleotide polymorphism was compared in HCV infected patients and healthy individuals.

Materials and Methods

Study Population

Patients and control individuals in this study were from Mashhad city, Northeast of Iran. All samples were recruited from those who were referred to Ghaem and Imam Reza academic teaching hospitals during a 12-month period starting from September 2011 to September 2012.
The case group included 67 patients with HCV (59 men and 8 women). HCV-infected patients were individuals positive for anti-HCV IgG with ELISA kit (Delaware Biotech., USA) and HCV RNA with RT-PCR method as described previously (Afshari et al., 2014).
Control subjects (73 HCV negative individuals; 32 men and 41 women) were selected. All the procedures were carried out according to the principles of the institutional guidelines and the study was approved by the Ethics Committee of Mashhad University of Medical Sciences.
A written informed consent was obtained from all subjects prior to recruitment. ALT levels were determined using Pars Azmoon Kit (Pars Azmoon, Iran) according to the manufacturers' instructions.

Extraction of Genomic DNA

Genomic DNA was obtained from peripheral blood samples and collected in EDTA tubes. The DNA was extracted using DNA Extraction Kit (Genet Bio, Korea).

Genotype Analysis

The genotypes of *p53* Arg72Pro polymorphism were determined using PCR-based restriction fragment length polymorphism (RFLP) method.
The forward primer used was 5'-ATCTACAGTCCCCCTTGCCG-3', whereas the reverse primer was 5'-GCAACTGACCGTGCAAGTCA-3' (Okada et al., 2001). Each PCR reaction mixture (30 µl) contained 0.4 mM of each primer, 1.5 mM $MgCl_2$, 0.4 mM dNTP, 0.4 U of *taq* DNA polymerase (Genet Bio, Korea), and 40 ng of genomic DNA in 10X reaction buffer. PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s.
The final extension was at 72°C for 7 min. After confirmation of an amplified fragment of the expected size (296 bp) on an agarose gel, 10 µl of PCR product was digested with 5 units of restriction enzyme *BstUI* (Fermentase, Germany) at 60°C for at least 3 h.
DNA fragments were checked through electrophoresis on a 3% agarose gel and stained with green viewer (Pars Tous, Iran).
The Arg allele is cleaved by *BstUI* and yields two small fragments (169 and 127 bp). The Pro allele was not cleaved by *BstUI* , resulted in a single 296-bp band.
The heterozygotes give three bands on gel electrophoresis (296, 169 and 127 bp) (Figure 1).
Although the HCV genotype frequencies in the patients of our study were determined previously, we randomly selected 30% of patients and genotyped them using genotype specific primers (Afshari et al., 2014).

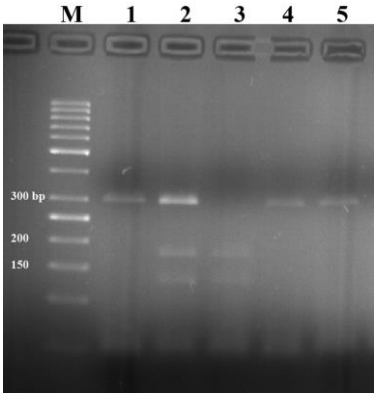

Figure 1. PCR products were digested by *BstUI* restriction enzyme. The Arg allele *BstUI* digestion showed 169 and 127 bp fragments (lane 3). The Pro allele was not cleaved by *BstUI*, resulted in a single 296-bp band (lanes 1, 4, 5). The heterozygotes showed three bands (296, 169 and 127 bp) on gel electrophoresis (lane 2); Lane M: 50 bp DNA size marker.

Statistical Analysis

Difference in gender, addiction, alcohol consumption, transfusion and tattoo operation between HCV patients and controls were evaluated using the chi-square test. The association between the *p53* polymorphism and HCV was determined using the logistic regression method to assess odds ratio (OR) and 95% confidence intervals (95% CI).

Difference between values were considered significant when a two-tailed *P* was <0.05. Statistical analysis was performed with SPSS 21.0 software (SPSS Inc., Chicago, IL).

Results

Descriptive Characteristics of Cases And Controls

The distribution of demographic data for all patients and controls is shown in Table 1.

Male patients were slightly more than females while females in the control group had a higher proportion (11.94% vs. 56.16%).

The mean age was 43.52 and 36.22 years in cases and controls, respectively. Predictably, HCV patients were significantly more likely to be addicts.

Table 1. Demographic characteristics of the study population

Characteristics	Patients	Controls	P-Value
Male: Female	59:8	32:41	<0.05
Age (years; mean ±S.D.)	43.52 ±10.52	36.22 ±12.49	<0.05
Addiction N	32	0	<0.05
Alcohol N	28	2	<0.05
Transfusion N	26	7	<0.05
Tatoo N	24	0	<0.05
ALT (U l-1, mean±S.D.)	41.15 ±41.36	25.97 ±21.63	<0.05
Increased ALT* N (%)	23	14	<0.05

*(>41 U. L-1 for men and >31 U.L-1 for women)

Distribution of P53 Codon 72 Genotypes Among Patients And Controls

As shown in Table 2, the frequencies of the Arg/Arg, Arg/Pro, and Pro/Pro genotypes among cases were, 26.86, 35.82 and 37.31%, respectively. In controls frequencies of Arg/Arg, Arg/Pro, and Pro/Pro were calculated as 32.87, 27.39 and 39.72%. We then analysed the distribution of Arg72Pro genotypes in patients compared to control group which showed no significant difference.

There was also no significant difference in allele frequencies. In addition, in a recessive model analysis of this position (Pro-Pro vs. Pro-Arg+Arg-Arg), Pro-Pro genotype was more common in controls (*P*=0.77; OR=0.90, 95% CI: 0.46 -1.79).

Genotype Distribution of HCV

The dominant genotypes of the virus in our population were 1a and 3a, and these results were in line with previous results that reported 1a and 3a as the most common genotypes in HCV patients (Afshari et al., 2014; Vossughinia et al., 2012).

Discussion and Conclusion

In addition to epigenetic factors and life style, some genetic factors contribute to cancers through different mechanisms.

P53 tumour suppressor gene is one of the most significant factors which can cause structural changes related to different kind of cancers (Hollstein et al., 1991). These changes could be observed in stem cells (genetic polymorphism) and somatic cells (mutation). Somatic mutation of p53 Arg72Pro in cancer, such as hepatocellular carcinoma, has been studied widely (Kawajiri et al., 1993; Papadakis et al., 2000; Själander et al., 1995). However, the probable relationship between p53 Arg72Pro gene polymorphism and HCV infection has not been studied in Iran. Several viral cancerous proteins react with p53 and can modulate its biological functions (Dobner et al., 1996; Friborg et al., 1999; Ko and Prives, 1996; Wang et al., 1994). It has been reported that in cervical cancer caused by human papillomavirus (HPV), an exon 4 polymorphism, which encodes for arginine in codon 72 (p53 Arg), confers more sensitivity to degradation by HPV E6 (Storey et al., 1998; Zehbe et al., 1999). Similar mechanism may be responsible for higher incidence of HCC in HCV infections. It has also been reported that NS3 protein of HCV has an interaction with p53. It has been found that natural set of NS3 and p53 aggregate in nucleus but mutant NS3 and p53 aggregate in cytoplasm (Ishido and Hotta, 1998; Muramatsu et al., 1997). The polymorphism of codon72 in p53 may affect NS3 aggregation and function. This process plays a role in virus proliferation, and may cause cell malignant transformation and deformation (Sakamuro et al., 1995). It is assumed that the core protein (central) of HCV is also included in malignant cell transformation (Moriya et al., 1998). It has been found that this protein reacts with p21 (Wang et al., 2000), which is a cell-cycle regulator and are induced by p53 (El-Deiry et al., 1993). P53 polymorphisms may also affect p21 induction and alter HCV proliferation and malignant hepatocytes transformation. It is worth noting that in cervical cancer caused by human papillomavirus, p53Arg is considered as the risky allele and this is inconsistent with present findings. This may be due to different reactions of p53 polymorphic protein with products of papillomavirus and HCV.

Table 2. Genotype distribution and allele frequencies of p53 codon 72 polymorphism in HCV patients and controls.

Codon 72 of *P53* gene	Controls N (%)	Patients N (%)	P value	OR	95% C.I.
Genotype					
Pro-Pro	29 (39.72)	25 (37.31)	Ref		
Pro-Arg	20 (27.39)	24 (35.82)	0.47	0.74	(0.33-1.66)
Arg-Arg	24 (32.87)	18 (26.86)	0.67	1.19	(0.53-2.69)
Pro-Pro	29 (39.72)	25 (37.31)	0.77	0.90	(0.46-1.79)
Arg-Arg/Pro-Arg	44 (60.29)	42 (62.68)			
Arg-Arg	24 (32.87)	18 (26.86)	0.43	0.75	(0.36-1.55)
Pro-Pro/Pro-Arg	49 (67.11)	49 (73.13)			
Allele Frequency					
Prolin	78 (53.42)	74 (55.22)	0.76	1.08	(0.67-1.72)
Arginin	68 (46.57)	60 (44.77)			

Since the dominant genotypes of HCV in Khorasan Razavi province and city of Mashhad were 1a and 3a (Afshari et al., 2014; Okada et al., 2001), in the current study only 20 samples were randomly genotyped for HCV types and the same results were achieved with a predominant 1a and 3a HCV genotypes. This may explain why the negative correlation between polymorphic p53 with HCV genotype was only observed in types 1a and 3a virus. It could be partly due to the fact that fewer numbers of cases were infected with other HCV genotypes. Consequently, structural differences of descriptive areas of HCV virus genotype may be included in p53 reaction. In women, other genetic or life style factors may influence HCV infection. In fact, as mentioned before, prevalence of HCV infection is significantly lower in men compared to women in this region (Shakeri et al., 2013). Leveri et al. did find any association between codon 72 genotypes and risk of cirrhosis and hepatocarcinoma in HCV patients (Leveri et al., 2004).In another study, it was shown that the Pro allele of the p53 Arg72Pro SNP has an increased risk for HCC in HBs Ag-negative subjects (Zhu et al., 2005). Okada et al. illustrated that at least in males, homozygosity for Pro in codon 72 of the p53 gene is one of the risk factors for infection with HCV genotype 1b (Okada et al., 2001). In addition, Anzola et al. showed that there is no significant correlation between codon 72 of p53 gene polymorphism and hepatocellular carcinoma in HCV patients (Anzola et al., 2003).

As our study has been done for the first time in Iran, further studies with larger sample size are required to explore this association.

Acknowledgments

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References

1. Afshari R., Nomani H., Zaniani F. R., Nabavinia M. S., Mirbagheri Z., Meshkat M., Gerayli S., Rostami S. and Meshkat Z. (2014) Genotype distribution of hepatitis C virus in Khorasan Razavi Province, Iran. Turkish Journal of Medical Sciences 44:656-660.
2. Anzola M., Cuevas N., López-Martí M., Saiz A., Burgos J. J. and Martí M. (2003) Frequent loss of p53 codon 72 Pro variant in hepatitis C virus-positive carriers with hepatocellular carcinoma. Cancer letters 193:199-205.
3. Chen F., Wang W. and El-Deiry W. S. (2010) Current strategies to target p53 in cancer. Biochemical Pharmacology 80:724-730.
4. Dobner T., Horikoshi N., Rubenwolf S. and Shenk T. (1996) Blockage by adenovirus Erorf6 of transcriptional activation by the p53 tumor suppressor. Science 272:1470-1473.
5. El-Deiry W. S., Tokino T., Velculescu V. E., Levy D. B., Parsons R., Trent J. M., Lin D., Mercer W. E., Kinzler K. W. and Vogelstein B. (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75:817-825.
6. Friborg J., Kong W.-p., Hottiger M. O. and Nabel G. J. (1999) p53 inhibition by the LANA protein of KSHV protects against cell death. Nature 402:889-894.
7. Goh A. M., Coffill C. R. and Lane D. P. (2011) The role of mutant p53 in human cancer. The Journal of Pathology 223:116-126.
8. Hagn F., Klein C., Demmer O., Marchenko N., Vaseva A., Moll U. M. and Kessler H. (2010) BclxL changes conformation upon

binding to wild-type but not mutant p53 DNA binding domain. Journal of Biological Chemistry 285:3439-3450.
9. Hollstein M., Sidransky D., Vogelstein B. and Harris C. C. (1991) p53 mutations in human cancers. Science 253:49-54.
10. Ishido S. and Hotta H. (1998) Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. FEBS Letters 438:258-262.
11. Kawajiri K., Nakachi K., Imai K., Watanabe J. and Hayashi S.-I. (1993) Germ line polymorphisms of p53 and CYP1A1 genes involved in human lung cancer. Carcinogenesis 14:1085-1089.
12. Ko L. J. and Prives C. (1996) p53: puzzle and paradigm. Genes and Development 10:1054-1072.
13. Kraemer K. H., Lee M. M. and Scotto J. (1987) Xeroderma pigmentosum: cutaneous, ocular, and neurologic abnormalities in 830 published cases. Archives of Dermatology 123:241-250.
14. Leontieva O. V., Gudkov A. V. and Blagosklonny M. V. (2010) Weak p53 permits senescence during cell cycle arrest. Cell Cycle 9:4323-4327.
15. Leveri M., Gritti C., Rossi L., Zavaglia C., Civardi E., Mondelli M. U., De Silvestri A. and Silini E. M. (2004) Codon 72 polymorphism of P53 gene does not affect the risk of cirrhosis and hepatocarcinoma in HCV-infected patients. Cancer letters 208:75-79.
16. Li T., Kon N., Jiang L., Tan M., Ludwig T., Zhao Y., Baer R. and Gu W. (2012) Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell 149:1269-1283.
17. Marcel V., Dichtel-Danjoy M.-L., Sagne C., Hafsi H., Ma D., Ortiz-Cuaran S., Olivier M., Hall J., Mollereau B. and Hainaut P. (2011) Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. Cell Death and Differentiation 18:1815-1824.
18. Minaguchi T., Kanamori Y., Matsushima M., Yoshikawa H., Taketani Y. and Nakamura Y. (1998) No evidence of correlation between polymorphism at codon 72 of p53 and risk of cervical cancer in Japanese patients with human papillomavirus 16/18 infection. Cancer Research 58:4585-4586.
19. Molchadsky A., Rivlin N., Brosh R., Rotter V. and Sarig R. (2010) p53 is balancing development, differentiation and de-differentiation to assure cancer prevention. Carcinogenesis 31:1501-1508.
20. Moriya K., Fujie H., Shintani Y., Yotsuyanagi H., Tsutsumi T., Ishibashi K., Matsuura Y., Kimura S., Miyamura T. and Koike K. (1998) The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nature Medicine 4:1065-1067.
21. Muramatsu S., Ishido S., Fujita T., Itoh M. and Hotta H. (1997) Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization. Journal of Virology 71:4954-4961.
22. Okada F., Shiraki T., Maekawa M. and Sato S. (2001) A p53 polymorphism associated with increased risk of hepatitis C virus infection. Cancer Letters 172:137-142.
23. Papadakis E., Dokianakis D. and Spandidos D. (2000) p53 codon 72 polymorphism as a risk factor in the development of breast cancer. Molecular Cell Biology Research Communications 3:389-392.
24. Sakamuro D., Furukawa T. and Takegami T. (1995) Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. Journal of Virology 69:3893-3896.
25. Sepahi S., Pasdar A., Ahadi M., Gerayli S., Rostami S. and Meshkat Z. (2014) Haplotype analysis of interleukin-10 gene promoter polymorphisms in chronic hepatitis C infection: a case control study. Viral Immunology 27:398-403.
26. Shakeri M. T., Nomani H., Mobarhan M. G., Sima H. R., Gerayli S., Shahbazi S., Rostami S. and Meshkat Z. (2013) The prevalence of hepatitis C virus in mashhad, iran: a population-based study. Hepatitis monthly 13.
27. Själander A., Birgander R., Athlin L., Stenling R., Rutegård J., Beckman L. and Beckman G. (1995) P53 germ line haplotypes associated with increased risk for colorectal cancer. Carcinogenesis 16:1461-1464.
28. Sjalander A., Birgander R., Hallmans G., Cajander S., Lenner P., Athlin L., Beckman G. and Beckman L. (1996) p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis 17:1313-1316.
29. Sotiropoulou P. A., Candi A., Mascré G., De Clercq S., Youssef K. K., Lapouge G., Dahl E., Semeraro C., Denecker G. and Marine J.-

C. (2010) Bcl-2 and accelerated DNA repair mediates resistance of hair follicle bulge stem cells to DNA-damage-induced cell death. Nature Cell Biology 12:572-582.
30. Srivastava S., Zou Z., Pirollo K., Blattner W. and Chang E. H. (1990) Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348:747.
31. Storey A., Thomas M., Kalita A., Harwood C., Gardiol D., Mantovani F., Breuer J., Leigh I. M., Matlashewski G. and Banks L. (1998) Role of a p53 polymorphism in the development of human papilloma-virus-associated cancer. Nature 393:229-234.
32. Vossughinia H., Goshayeshi L., Bayegi H. R., Sima H., Kazemi A., Erfani S., Abedini S., Goshayeshi L., Ghaffarzadegan K. and Nomani H. (2012) Prevalence of hepatitis C virus genotypes in Mashhad, Northeast Iran. Iranian Journal of Public Health 41:56-61.
33. Wang F., Yoshida I., Takamatsu M., Ishido S., Fujita T., Oka K. and Hotta H. (2000) Complex formation between hepatitis C virus core protein and p21Waf1/Cip1/Sdi1. Biochemical and Biophysical Research Communications 273:479-484.
34. Wang X. W., Forrester K., Yeh H., Feitelson M. A., Gu J.-R. and Harris C. C. (1994) Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proceedings of the National Academy of Sciences 91:2230-2234.
35. Zehbe I., Voglino G., Wilander E., Genta F. and Tommasino M. (1999) Codon 72 polymorphism of p53 and its association with cervical cancer. The Lancet 354:218-219.
36. Zhao T. and Xu Y. (2010) p53 and stem cells: new developments and new concerns. Trends in Cell Biology 20:170-175.
37. Zhu Z.-Z., Cong W.-M., Liu S.-F., Xian Z.-H., Wu W.-Q., Wu M.-C., Gao B., Hou L.-F. and Zhu G.-S. (2005) A p53 polymorphism modifies the risk of hepatocellular carcinoma among non-carriers but not carriers of chronic hepatitis B virus infection. Cancer letters 229:77-83.
38. Ziech D., Franco R., Georgakilas A. G., Georgakila S., Malamou-Mitsi V., Schoneveld O., Pappa A. and Panayiotidis M. I. (2010a) The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. Chemico-Biological Interactions 188:334-339.
39. Ziech D., Franco R., Pappa A., Malamou-Mitsi V., Georgakila S., Georgakilas A. G. and Panayiotidis M. I. (2010b) The role of epigenetics in environmental and occupational carcinogenesis. Chemico-Biological Interactions 188:340-349.
40. Ziech D., Franco R., Pappa A. and Panayiotidis M. I. (2011) Reactive Oxygen Species (ROS)—Induced genetic and epigenetic alterations in human carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 711:167-173.

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Research Article

Geometric Mean of 5S rRNA and MiR-16 as a Suitable Normalizer in Esophageal Cancer

Samaneh Khazaei[1], Sedigheh Gharbi[2], Seyed Javad Mowla[3*]

[1] *Division of Genetics, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran*
[2] *Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran*
[3] *Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a deadly cancer with poor prognosis. In this regard, early diagnosis is of vital importance to cure the tumor in its early stages. Novel cancer diagnostic and therapeutic approaches have been recently introduced based on microRNAs (miRNAs). Also, accurate normalization using appropriate reference genes is a critical step in miRNA expression studies. In this study, we aimed to identify appropriate reference genes for miRNA quantification in serum samples of ESCC. In this case and control experimental study, two statistical algorithms including GeNorm and NormFinder were used to evaluate the suitability of miR-16 and 5S rRNA and their geometric mean as reference genes. Then, relative expression of miR-451 and miR-24 were evaluated while different normalizer including miR-16, 5S rRNA and their geometric mean were applied. Both GeNorm and NormFinder analyses showed that geometric mean of miR-16 and 5S rRNA is the most stable reference gene in these samples. Also, our data showed that choosing an inappropriate normalizer could change the relative expression of target genes of miR-451 and miR-24 in ESCC samples which emphasize on the importance of selecting a reliable internal control in expression analyses. We demonstrated that geometric mean of two reference genes could increase the reliability of normalizers and also by using geometric mean as reference gene, relative expression of different target is closer to reality.

***Keywords*:** Esophageal cancer, MicroRNA, qRT-PCR, Reference genes

Introduction

Esophageal cancer is one of the most common malignancies in the world which ranked eighth in cancer incidence and sixth in cancer mortality (Zhang, 2013). There are two major types of esophageal cancer, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (Xu et al., 2012). Golestan province in northern Iran is known as an area with a very high incidence of esophageal cancer (Islami et al., 2009). ESCC is a deadly cancer with poor prognosis which its early diagnosis is of vital importance to cure the tumor in its early stages (Fitzmaurice et al., 2015). Novel cancer diagnostic and therapeutic approaches have been recently introduced based on miRNAs (Monroig and Calin, 2013). MiRNAs are short RNA molecules that bind (generally) to 3' UTR sequences of target messenger RNAs (mRNAs), thereby modulating their expression patterns. This modulated gene expression is regulated either as translational repression (Lai, 2002), or mRNA degradation (Engels and Hutvagner, 2006).

MiRNAs play major roles in governing diverse biological processes such as differentiation, proliferation, and apoptosis (Chen et al., 2004; Croce and Calin, 2005). Individual miRNAs have oncogenic and tumor suppressor functions (Esquela-Kerscher and Slack, 2006), and aberrant miRNA expression has been implicated in many malignancies.

Several methods have been employed in miRNA expression studies including Northern blotting, microarrays, reverse transcription-qPCR (RT-qPCR), and sequencing. RT-qPCR possesses advantages in terms of high sensitivity, wide dynamic range and low template requirements (Pritchard et al., 2012). Appropriate normalization of RT-qPCR data using stably expressed reference genes is critical to ensure accurate and reliable results, because data normalization with an inappropriate reference gene would lead to twisted and biased results (Chang et al., 2010; Das et al., 2016; Ferdous et al., 2015). So, in order to achieve

Corresponding authors E-mail:
* *sjmowla@modares.ac.ir*

accurate, reproducible and biologically relevant miRNA RT-qPCR data and also to remove any non-biological sample-to-sample variation, appropriate reference genes should be selected in each study design (Liu et al., 2014).

A candidate reference gene should meet certain criteria before being applied as a reliable normalizer in expression studies. These criteria include having comparable length, expression level and quantification efficiency to the target gene, and most importantly displaying an invariable expression level across all samples under study (DAS et al., 2016; Schaefer et al., 2010; Shen et al., 2011).

The aim of this study was to investigate the suitability of two widely used reference genes in serum samples of patients with esophageal cancer. An additional goal of this study was to assess the impact of exploiting different reference genes on miRNA quantification in ESCC disease.

In this regard, the stability of miR-16 and 5S rRNA was evaluated in serum samples of ESCC. Then, different algorithms were used to determine the most stable reference genes in these samples. Finally, miR-451 and miR-24 expression were normalized to miR-16 and 5S rRNA and also to their geometric mean.

Materials and Methods

Clinical Sample Collection

39 serum samples from patients with ESCC and 39 serums from normal individuals were provided by Atrak Hospital (Golestan University of Medical Sciences, Iran). The histo-pathological characteristics of the ESCC patients are summarized in Table 1.

Ethics Statement

This study was reviewed and approved by the Ethical Committee of Golestan University of Medical Sciences. All samples were collected according to the institutional policies and patients informed consent.

RNA Extraction From Esophageal Serum

All serum samples were first centrifuged for 5 minutes at 3000 rpm to remove any cell contamination or cell debris. Next, in order to remove protein contamination, all serum samples were treated with Proteinase K (Fermentas, Lithuania) for 3 hours at 54°C. RNA was then extracted from serum samples with TRIzol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. The resultant RNA pellets were dissolved in 20 ul of RNase free water and were kept in -80°C for further analyses.

miR-451 and miR-24 Quantification by Quantitative RT-PCR

Due to the presence of inhibitors in serum, as recommended by Gharbi and colleagues, different volumes of RNA samples should be tested for cDNA synthesis (Gharbi et al., 2014).

Firstly, 5 µl of total RNA sample was polyadenylated by poly (A) polymerase and then 1.5 µl of poly-A-adenylated RNA which has the least amount of inhibitors, was subjected to reverse transcription reaction in a total volume of 10 µl. This reaction was performed using RT-PCR kit, miR-451 and miR-24-specific primer bearing a 3' universal tag (ParsGenome, Iran).

Real-time PCR was performed using each miRNA specific primers, universal reverse primer (complement to the universal tag added in RT-PCR step to the end of each miRNA), and SYBR Green Premix (ParsGenome, Iran) using Step One real-time PCR machine (Applied Biosystems, USA).

Data Analyses

qRT-PCR data analyses were performed using ΔΔCq (quantitation cycle) method and gene expressions were normalized to the expression levels of different reference genes in each experiment. All experiments were performed in duplicates.

The t test statistic was used for comparison of the distribution of the reference genes expression between the control and patient groups (significance level=0.05). Data was analyzed using SPSS 16.0 (SPSS Inc, USA) and GraphPad Prism 6 softwares.

GeNorm (GenEX software) and NormFinder (GenEX software) were used to analyze the stability of the examined reference genes in all tested samples including cases and controls. The GeNorm software ranks the tested genes based on their expression stability (M value) and introduces the two most stable reference genes among all tested genes.

The M value describes the mean pairwise variation of a candidate gene compared with all other candidate genes. The stability ranking of each candidate gene was then determined by stepwise exclusion of the gene with the highest M value, followed by recalculation of average expression stability for the remaining genes until the two most stable genes were found.

The NormFinder uses a different mathematical model compared to GeNorm and takes into account the intragroup and intergroup variation. In addition, NormFinder calculates a stability value for each reference gene and ranks the candidate genes based on accumulated standard deviation (Acc SD).

Table 1. Histopathological characteristics of the patients

Age (year)	Sex		Tumor grade			Normal esophagus				Mucosal biopsy	
	Female	Male	Low	Moderate	High	No changes	Mild esophagitis	High grade squamous dysplasia	Low grade squamous dysplasia	Mild chronic gastric	*H. pylori* associated chronic gastric
62.77%	66.6 %	33.4 %	77.8 %	16.7 %	5.5%	55.5 %	33.5 %	5.5 %	5.5 %	76.7 %	23.3%

Results

Expression Pattern of Candidate Reference Genes In Serum

The average Cq values of both reference genes and their geometric mean are shown in table 2. As shown in this table, SD of reference genes was decreased when geometric mean of two reference genes is used.

Table 2. Descriptive statistical values of Cycle of quantification of reference genes in 39 tested samples

Rank	Gene	Min	Max	Average	Median	SD
1	miR-16	26.91	34.95	30.93	31.67	1.94
2	5S rRNA	23.42	29.15	26.28	26.30	1.62
3	Geometric mean	26.22	29.78	28	29.08	1.41

Expression Levels of Candidate Reference Genes In Patient And Healthy Groups

None of the reference genes were affected by the disease state since observed Cq values did not show any significant difference between healthy and patient groups (Fig.1). In addition, the result of t test with P>0.05 for both reference genes indicated that the tested populations of patients and controls have equal variance. This is an essential step before evaluating the stability of reference genes.

Expression Stability of Candidate Reference Genes

Expression stability of the candidate genes were assessed using comparison of their standard deviation and also using two algorithms of GeNorm and NormFinder as shown in table 2. miR-16 had the most variation and geometric mean had the last SD in these samples. GeNorm analysis showed that geometric mean and 5S rRNA had the same stability in these samples (Fig 2), but NormFinder analyses ranked geometric mean as the most stable and adequate reference genes in these samples (Fig 3). In total, our analyses revealed that the stability of the geometric mean normalizer is significantly higher than each candidate individually.

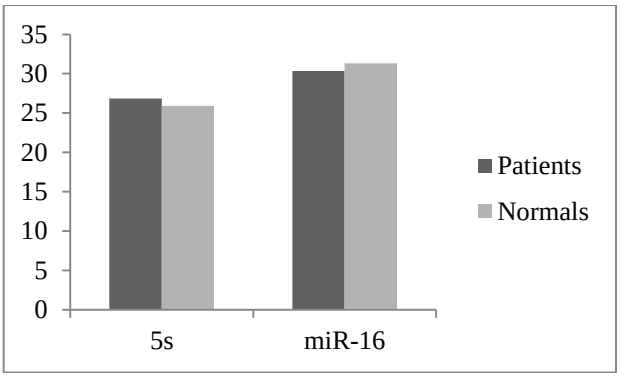

Figure 1. Cq values of candidate genes in tested samples. No differences were found between the control and patient groups (P>0.05). Cq; Cycle of Quantification.

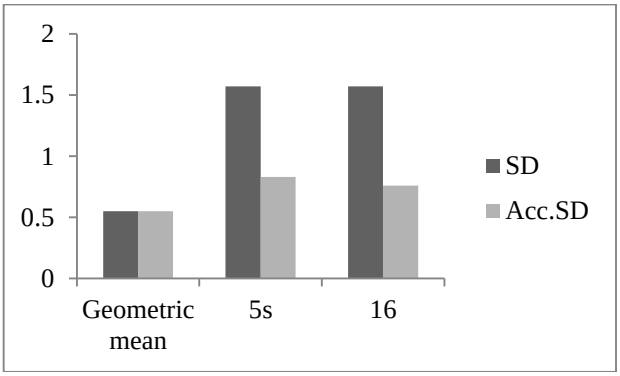

Figure 2. Normfinder analysis of 5S rRNA and miR-16 after applying geometric mean as a separate normalizer. The expression stability of each candidate is shown by SD in NormFinder. The lower value of SD represents higher gene stability. SD; Standard Deviation.

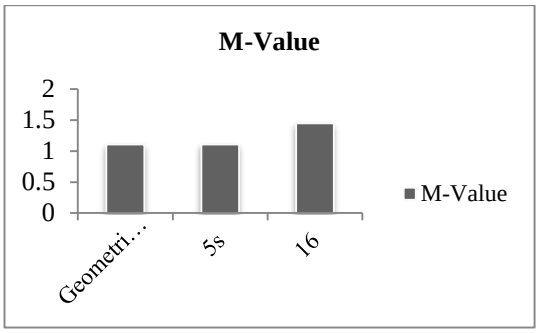

Figure 3. GeNorm analysis of 5S rRNA after applying geometric mean as a separate normalizer.

Target Gene Expression Normalized With Different Reference Genes

We evaluated the expression level of two target genes including miR-451 and miR-24 in these samples and normalized them with miR-16, 5S rRNA and their geometric mean as reference genes (Fig. 4 -5). As shown in these figures, relative expression and P-value of target genes is different when normalized with different reference genes and to their mean. In case of miR-451, the fold changes were 1.92, 0.94 and 6.65 normalized to 5S rRNA, miR-16 and geometric mean respectively. This results showed that by applying the geometric mean as the most stable normalizer instead of miR-16, the fold change was remarkably changed from 0.94 (P value= 0.69) to 6.65 (P value=0.07). The same results obtained in the case of miR-24 as well which means normalization with different normalizer could change the fold change in an opposite direction and also it can influence the experiment significance.

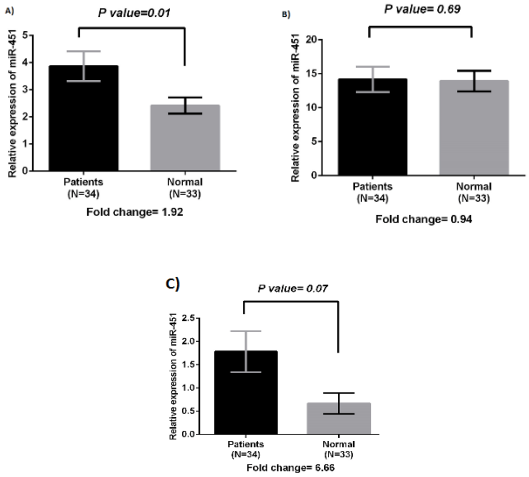

Figure 4. Relative expression of miR-451 in serum samples with ESCC compared to normal samples. miR-451 relative expression when normalized with: A) Geometric mean of miR-16 and 5S rRNA (Fold change= 1.92; P-value=0.01). B) miR-16 (Fold change=0.94; P-value=0.69). C) 5S rRNA (Fold change= 6.65; P-value= 0.07).

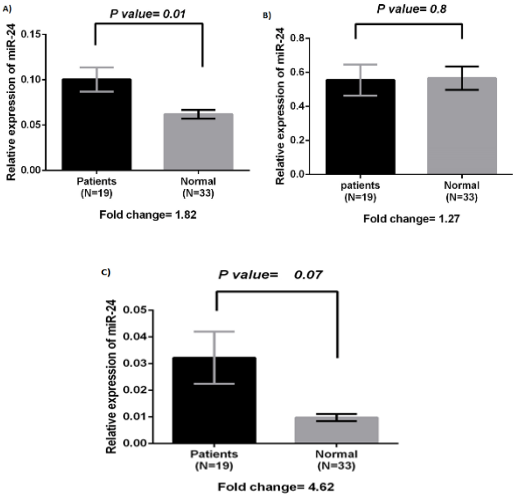

Figure 5. Relative expression of miR-24 in serum samples with ESCC compared to normal samples. miR-24 relative expression when normalized with: A) Geometric mean of miR-16 and 5S rRNA (Fold change= 1.82; P-value=0.01). B) miR-16 (Fold change=1.27; P-value=0.8). C) 5S rRNA (Fold change= 4.62; P-value= 0.07)

Discussion

MicroRNAs represent an important new class of biomarkers with critical role in post-transcriptional gene regulation, where their aberrant expressions have been observed in various diseases states. These tiny molecules regulate a large number of important biological processes including early development, cell proliferation, differentiation, apoptosis, fat metabolism, and oncogenesis (Lu et al., 2005). Reverse transcription quantitative real-time PCR (RT-qPCR) is the most sensitive and reliable method of choice which has been widely used in microRNA expression studies. To achieve accurate and reproducible RT-qPCR data, appropriate normalization is critical to correct technically, non-biological variations (Peltier and Latham, 2008). More recently, it was advised to validate the suitability of a panel of internal control genes in each sample. Inappropriate normalization of data can lead to incorrect conclusion and can conceal or magnify biologically meaningful changes of microRNAs (Vandesompele et al., 2002). Vansesompele et al proposed to evaluate the suitability of one to three commonly reference genes for each specific study (Lu et al., 2005). Here, 5S rRNA and miR-16, two commonly used reference genes in microRNA studies of serum samples, were selected and their expression was evaluated using q-RT PCR in serum samples of esophagus cancer.

As a pre-requirement of our analysis, we showed that

expression pattern of miR-16 and 5S rRNA is the same in the case and control groups. Lack of difference in this pattern showed that the tested candidate genes are not influenced by physiological state of the disease.

Data analyses on these samples using GeNorm and NormFinder showed that the geometric mean of miR-16 and 5S rRNA has the least variation compared to each of the mentioned reference genes individually. This result is consistent with the results of Vandesompele et al which showed the geometric mean of three reference genes results in more accurate data (Vandesompele et al., 2002). Gharbi and colleagues in their studies on serum samples showed that geometric mean of two reference genes is the most stable normalizer. They also showed that geometric mean of two reference genes represents the least variation even when one of these reference genes has the least stability in the samples (Gharbi et al., 2015). Then, expression level of miR-24 and miR-451 was evaluated in serum samples of esophageal cancer compared to control group. The normalization factor was 5S rRNA, miR-16 and their geometric mean. We observed that miR-24 and miR-451 were significantly over expressed when the data was normalized to 5S rRNA and geometric mean. But applying miR-16 as normalizer, the expression level between case and control group was not significant. Up regulation of miR-24 and miR-451 in biological fluids of esophageal cancer has been reported in some studies (Gu et al., 2014; Murata et al., 2013; Zhu et al., 2014). This variation in the result reveals the importance of selecting a reliable internal control in expression studies. Lim and colleagues assessed the suitability of a panel of internal controls in neuronal differentiation. They showed that normalization to an unsuitable reference gene could under-estimate the up regulation of miR-125 and miR-211 (Lim et al., 2011).

References

1. Chang K. H., Mestdagh P., Vandesompele J., Kerin M. J. and Miller N. (2010) MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC Cancer 10:173.
2. Chen C.-Z., Li L., Lodish H. F. and Bartel D. P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83-86.
3. Croce C. M. and Calin G. A. (2005) miRNAs, cancer, and stem cell division. Cell 122:6-7.
4. Das M. K., Andreassen R., Haugen T. B. and Furu K. (2016) Identification of Endogenous Controls for Use in miRNA Quantification in Human Cancer Cell Lines. Cancer Genomics and Proteomics 13:63-68.
5. Engels B. M. and Hutvagner G. (2006) Principles and effects of microRNA-mediated post-transcriptional gene regulation. Oncogene 25:6163-6169.
6. Esquela-Kerscher A. and Slack F. J. (2006) Oncomirs—microRNAs with a role in cancer. Nature Reviews Cancer 6:259-269.
7. Ferdous J., Li Y., Reid N., Langridge P., Shi B. J. and Tricker P. J. (2015) Identification of reference genes for quantitative expression analysis of microRNAs and mRNAs in barley under various stress conditions. PloS One 10:e0118503.
8. Fitzmaurice C., Dicker D., Pain A., Hamavid H., Moradi-Lakeh M., MacIntyre M. F., Allen C., Hansen G., Woodbrook R. and Wolfe C. (2015) The global burden of cancer 2013. JAMA Oncology 1:505-527.
9. Gharbi S., Mirzadeh F., Khatrei S., Soroush M. R., Tavallaie M., Nourani M. R. and Mowla S. J. (2014) Optimizing microRNA quantification in serum samples. Journal of Cell and Molecular Research 6:52-56.
10. Gharbi S., Shamsara M., Khateri S., Soroush M. R., Ghorbanmehr N., Tavallaei M., Nourani M. R. and Mowla S. J. (2015) Identification of reliable reference genes for quantification of microRNAs in serum samples of sulfur mustard-exposed veterans. Cell Journal (Yakhteh) 17:494.
11. Gu Y.-Q., Gong G., Xu Z.-L., Wang L.-Y., Fang M.-L., Zhou H., Xing H., Wang K.-R. and Sun L. (2014) miRNA profiling reveals a potential role of milk stasis in breast carcinogenesis. International Journal of Molecular Medicine 33:1243-1249.
12. Islami F., Kamangar F., Nasrollahzadeh D., Møller H., Boffetta P. and Malekzadeh R. (2009) Oesophageal cancer in Golestan Province, a high-incidence area in northern Iran–A review. European Journal of Cancer 45:3156-3165.
13. Lai E. C. (2002) Micro RNAs are complementary to 3 [variant prime] UTR sequence motifs that mediate negative post-transcriptional regulation. Nature Genetics 30:363.

14. Lim Q., Zhou L., Ho Y., Wan G. and Too H. (2011) snoU6 and 5S RNAs are not reliable miRNA reference genes in neuronal differentiation. Neuroscience 199:32-43.
15. Liu X., Zhang L., Cheng K., Wang X., Ren G. and Xie P. (2014) Identification of suitable plasma-based reference genes for miRNAome analysis of major depressive disorder. Journal of Affective Disorders 163:133-139.
16. Lu J., Getz G., Miska E. A., Alvarez-Saavedra E., Lamb J., Peck D., Sweet-Cordero A., Ebert B. L., Mak R. H. and Ferrando A. A. (2005) MicroRNA expression profiles classify human cancers. Nature 435:834-838.
17. Monroig P. d. C. and Calin G. A. (2013) MicroRNA and epigenetics: diagnostic and therapeutic opportunities. Current Pathobiology Reports 1:43-52.
18. Murata K., Furu M., Yoshitomi H., Ishikawa M., Shibuya H., Hashimoto M., Imura Y., Fujii T., Ito H. and Mimori T. (2013) Comprehensive microRNA analysis identifies miR-24 and miR-125a-5p as plasma biomarkers for rheumatoid arthritis. PloS One 8:e69118.
19. Peltier H. J. and Latham G. J. (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14:844-852.
20. Pritchard C. C., Cheng H. H. and Tewari M. (2012) MicroRNA profiling: approaches and considerations. Nature Reviews Genetics 13:358-369.
21. Schaefer A., Jung M., Miller K., Lein M., Kristiansen G., Erbersdobler A. and Jung K. (2010) Suitable reference genes for relative quantification of miRNA expression in prostate cancer. Experimental and Molecular Medicine 42:749-758.
22. Shen Y., Li Y., Ye F., Wang F., Wan X., Lu W. and Xie X. (2011) Identification of miR-23a as a novel microRNA normalizer for relative quantification in human uterine cervical tissues. Experimental and Molecular Medicine 43:358-366.
23. Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. and Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: research0034. 0031.
24. Xu Q., Ma P., Hu C., Chen L., Xue L., Wang Z., Liu M., Zhu H., Xu N. and Lu N. (2012) Overexpression of the DEC1 protein induces senescence in vitro and is related to better survival in esophageal squamous cell carcinoma. PloS One 7:e41862.
25. Zhang Y. (2013) Epidemiology of esophageal cancer. World Journal of Gastroenterology 19.
26. Zhu C., Ren C., Han J., Ding Y., Du J., Dai N., Dai J., Ma H., Hu Z. and Shen H. (2014) A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. British Journal of Cancer 110:2291-2299.

Research Article

Protective Effect of Diosgenin against H_2O_2-Induced Oxidative Stress on H9C2 Cells

Samaneh Jamshidi[1], Mehrdad Lahouti[1*], Mohammad Taher Boroushaki[2], Ali Ganjeali[1], Ahmad Ghorbani[2], Mehdi Bihamta Toosi[2]

[1] *Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Iran*
[2] *Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Science, Mashhad, Iran*

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Abstract

Diosgenin is an important compound in pharmaceutical industry. It has various effects such as hypocholesterolemic action or antioxidant activity in HIV infected patients. Biological oxidation pathways are involved in causing or aggravating heart disease. This study investigated the potential protective effect of diosgenin on cell viability and antioxidant defenses of cultured H9C2 cells submitted to oxidative stress induced by H2O2. Viability of cells exposed to H2O2 was detected by MTT assay. The generation of ROS and hydrogen peroxide release after H2O2 were detected using the fluorescent probe H2DCF-DA. The lipid peroxidation product i.e. MDA formation was estimated by assessing the levels of thio-barbituric acid reactive substances (TBARS) using spectrophotometry. SOD activity was assayed with NWLSS (TM) Superoxide Dismutase (SOD) activity assay kit. Pretreatment of cells with 3-25 µM of diosgenin for 24 h before applying H2O2 completely prevented cell damage and significantly enhanced viability of H9C2 cells. Increased ROS induced by H2O2 was dose dependently prevented when cells were pretreated for 24 h with diosgenin. The level of the lipid peroxidation was significantly higher in H9C2 cells exposed to H2O2 as compared to the control and cells pretreated with diosgenin. SOD activity in cells treated with diosgenin significantly decreased compared with cells exposed to H2O2. These results show that treatment of H9C2 cells with diosgenin (3-25 µM) confers a significant protection against oxidative stress.

***Keywords*:** Diosgenin, H9C2 cells, Oxidative stress, MDA, Cell viability

Introduction·

Diosgenin is a steroidal sapogenin belonging to the group of triterpenes. It is found in several plants including fenugreek (Trigonella foenum graecum), the roots of the wild yam (Dioscorea villosa) and Costus speciosus (Attele et al., 1999; Liu et al., 2005). Steroidal sapogenins are secondary metabolites and their biosynthetic precursors are sterols, especially cholesterol. They are mainly found as glycosides called steroidal saponins, which constitute a structurally diverse class of natural products and are one of the major components in traditional Chinese medicines (Attele et al., 1999a; Liu et al., 2005). Diosgenin is an important compound in pharmaceutical industry as a natural source of steroidal hormones (Liu et al., 2005; Roman et al., 1995). It has various effects, such as hypocholesterolemic action or antioxidant activity in HIV infected patients (Accatino et al., 1998; Kim et al., 2012; Turchan et al., 2003).

Diosgenin has anticancer effects against a wide variety of tumor cells, including colorectal cancer, breast cancer, osteosarcoma and leukemia (Corbiere et al., 2003; Liu et al., 2005; Srinivasan et al., 2009; Wang et al., 2004). Other researchers have reported that it has estrogenic effects (Aradhana et al., 1992). Diosgenin acts as a megakaryocytic differentiation inducer and could cause changes in lipoxygenase activities in human erythroleukemia cells. Five lipoxygenase activating protein (FLAP), and leukotriene A4 (LTA4) hydrolase gene expression during megakaryocytic differentiation induced by diosgenin (Beneytout et al., 1995; Corbiere et al., 2003; Wei et al., 2001). It induces p53-mediated cell cycle G_1 arrest and apoptosis in osteosarcoma cells (Moalic et al., 2001). It is necessary to study the biochemical and cellular mechanisms of action of this natural product. Hydrogen peroxide is a physiological component of living cells and is uninterruptedly produced via various cellular pathways. The intracellular concentration of H2O2 is strongly controlled by enzymatic and nonenzymatic antioxidant systems.

Corresponding authors E-mail:
* *mlahouti@um.ac.ir*

Intracellular steady-state concentrations of H2O2 above 1 µM are considered to cause oxidative stress inducing growth arrest and cell death (Antunes and Cadenas, 2001; Stone and Yang, 2006).

Oxidative stress that resulting from increased production of free radicals and reactive oxygen species, and/or a decrease in antioxidant defense, leads to damage of biological macromolecules and disruption of normal metabolism and physiology and also pathologies, such as cancer and neurological disorders, as well as in ageing (Bernabucci et al., 2002; Trevisan et al., 2001).

The role of free radicals, reactive oxygen species, and antioxidants in the etiology of chronic diseases, including cardiovascular disease, lung disease, cancer, diabetes, renal ischemia, atherosclerosis, pulmonary pathological states, inflammatory diseases and others, has stimulated research in recent years (Santanam et al., 1998; Trevisan et al., 2001)

It is widely accepted that an excess of ROS is toxic and damages cell components including nucleic acids, proteins and lipids (Pizarro et al., 2009; Thannickal and Fanburg, 2000).

Lipids are important component of the cell membrane. Lipid peroxidation is implicated in the pathogenesis of a number of diseases and clinical conditions (P et al., 2013) which include diabetes, adult respiratory distress syndrome, premature birth disorder, aspects of shock, Parkinson's disease, Alzheimer's disease, pre-eclampsia and eclampsia, various chronic inflammatory conditions, ischaemia, reperfusion mediated injury to organs which include the heart, brain and the intestine, atherosclerosis, organ injury which is associated with shock and inflammation, fibrosis, cancer, inflammatory liver injury, anthracycline induced cardiotoxicity, silicosis and pneumoconiosis (Davi et al., 2005; Riley, 1994; Yagi, 1987).

The lipid peroxidation product, malondialdehyde (MDA), is commonly used as a measure of the oxidative stress in cells. Lipid peroxidation occurs when the hydroxyl radicals, possibly oxygen, react with the unsaturated lipids of the bio-membranes, resulting in the generation of lipid peroxide radicals (ROO•), lipid hydroperoxide (ROOH) and fragmentation products such as MDA (Uchida et al., 1999). This aldehyde is a highly toxic molecule and it should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been referred as a potentially mutagenic and atherogenic agent (Lores Arnaiz et al., 1998; Ueda et al., 1998). Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways. One of the primary antioxidant enzymes in cells that is thought to be necessary for life in all oxygen metabolizing cells is superoxide dismutase (SOD). The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O2) (Peskin and Winterbourn, 2000).

In this study, we investigated the effect of diosgenin on the proliferation rate and diosgenin ability to protect H9C2 cells from cell death when exposed to oxidative stress induced by hydrogen peroxide.

Materials and Methods

Cell Culture and Drug Treatment

H9C2 cells were obtained from Razi Vaccine and Serum Research Institute and were cultured in RPMI (Gibco) supplemented with 20% fetal bovine serum (Gibco) and 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were cultured at 37°C in a humidified chamber with 95% air and 5% CO_2. All experiments were performed in plastic tissue culture flasks. H9C2 cells were seeded in 24 or 96 well plates. After plating, cells were allowed to adhere overnight and were then treated with chemicals. Diosgenin was purchased from Sigma Chemical Co (D1634-5G). Diosgenin (10 mg) was dissolved in 2 ml of ethanol (12000 µM) and mixed with fresh medium to achieve the desired concentration (0, 1, 3, 6, 12, 25, 50, 100 and 200 µM) . The maximum final ethanol concentration in cultures was 0.7%, which did not alter cell growth and cell cycle measurements when compared with untreated control cells.

Determination of Cell Viability (MTT Assay)

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay. The cells were seeded in 96-well plates at a density of 5×10^3 cells/well and after 48 h, they were treated with various concentrations of diosgenin (0–200 μM) for 24h. After the exposure period, media were removed. MTT solution in phosphate-buffered saline (PBS, 5mg/mL) was added to a final concentration of 0.05% for 1 h, thereby allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed and formazan crystals were dissolved in 200 µl of dimethylsulphoxide. Formazan production was measured by the absorbance at 545 nm using a microplate reader (BioRad Laboratories, CA, USA). Viability results were expressed as percentages.

The percentage of cell viability was calculated by dividing the mean absorbance of each treatment to the mean absorbance of its controls multiply by 100.

Determination of Diosgenin Effect on Viability of H9C2 Cells Exposed to H_2O_2

Cells were planted into 96-well plates. After incubation for 48 hours, the medium was replaced with fresh medium with various concentrations of diosgenin (0–50 µM) for 24h .
Then, the medium was changed and incubated with or without H2O2 at indicated concentration (200 µM) for 1h. Six wells were included in each concentration.
At the end of treatment, 10 µl MTT was added and incubated for 1 h. Then the medium was discarded carefully and 200 µl DMSO was added. Absorbance was recorded at 545 nm with Universal Microplate Reader.
All experiments were performed in triplicateS. The mean percentage of cell death was calculated as follow:
% inhibition = (A545 of control _ A545 of treated cells)/A545 of control cells × 100%.

Measurement of ROS

Level of intracellular ROS was measured using the fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (H2DCF-DA). Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells/well and after 48 h, they were treated with various concentrations of diosgenin (0–50 µM).
After 24 h incubation, DMEM was replaced by PBS and the cells were treated with 1µM CM-H2DCFDA for 30 min at 37°C in darkness (added from a 20 mM stock solution in dimethyl sulphoxide).
H2DCFDA diffuses across cell membranes, where acetates migrate via intracellular esterases. Oxidation of H2DCFDA occurs almost exclusively in the cytosol, thereby generating a fluorescent response proportional to ROS generation.
After loading the dye, cells were washed in Locke's buffer and fluorescence was measured at a 488 nm excitation wavelength and an emission wavelength of 510 nm, using a Perkin-Elmer Victor 3 fluorometer.

Estimation of MDA

MDA was estimated by assessing the levels of Thio- Barbituric Acid Reactive Substances (TBARS). The TBARS assay was performed by using MDA equivalents which were derived from tetra-ethoxy-propane. MDA was identified as a product of lipid peroxidation which reacted with TBA to give a pink coloured species that gave an absorbance at 532 nm.
Cells were seaded in 12-well plates. 48 hours after incubation, the medium was replaced with fresh medium with various concentrations of diosgenin (3–25 µM) for 24h. Then, the medium was changed and incubated with H2O2 at indicated concentration (200 µM) for 1h.
Afterwards, media was transferred to a fresh tube and the scraped cells with 1 ml TCA was added to the tube and the mixture was centrifuged at 13000 rpm for 5 min.
The method involved heating of the separated supernatant of the treated cells with the TBA reagent which contained Tri-chloro Acetic acid (TCA) (1.5 %) and Thio-Barbituric Acid (TBA)(0.7 %).
After cooling the solution, it was centrifuged at 2000 rpm and the precipitate was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained untreated cells.

SOD Activity Measurement

Superoxide Dismutase (SOD) Activity was assessed by NWLSS (TM) kit which is a sensitive kit using WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.
Cells were planted in 12-well plates. 48 hours after incubation, the medium was replaced with fresh medium with various concentrations of diosgenin (3–25 µM) for 24h. Then, the medium was changed and incubated with H2O2 at indicated concentration (200 µM) for 1h.
Afterwards, media was transferred to a fresh tube, the cells were scraped with cold Tris/HCl 0.1M, pH 7.4 containing 0.5 % Triton X-100, 5mM β-ME, and 0.1 mg/ml PMSF. Cell lysate was centrifuged at 14000 rpm for 5 minutes at 4°C.
Then, the supernatant was transferred to a tube which contains total SOD activity from cytosol and mitochondria. 40 µl of supernatant was poured into 18 tubes and 920 µL of assay buffer was added to each tube.
The solutions were mixed and incubated for 5 minutes. Then, 40 µl Hematoxylin Reagent added to start the reaction. The mixture was vortexed quickly and the absorbance at 560 nm was measured. All experiments were performed in triplicates.

Statistical Analysis

Data were expressed as mean ± SEM. For statistical analysis, one-way analysis of variance (ANOVA) with Tukey–Kramer post hoc test for multiple comparisons were used. P value ≤ 0.05 was considered statistically significant.

Research Article

Results

Effect of Diosgenin on The Growth of H9C2 Cells

To determine diosgenin effect on cell viability , H9C2 cells were treated with diosgenin (0–200 µM). Cell viability was evaluated based on the ability of cells to exclude trypan blue.

Diosgenin induced a marked dose-dependent diminution of cell viability as early as 24 h, indicating that the proliferation potential of cells was impaired. After cells were treated with diosgenin, marked morphological changes of cell apoptosis were found (Figure 1).

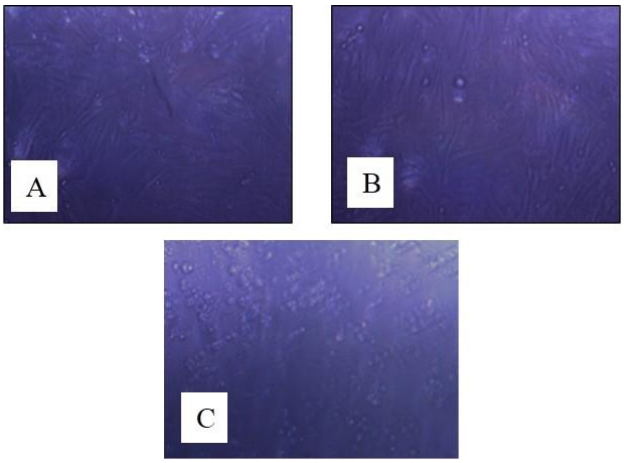

Figure 1. Cytotoxic Effect of diosgenin in H9C2 cells. After cells were treated with diosgenin (0–200 µM) for 24 h, marked morphological changes of cell apoptosis were found. . A: H9C2 cells cultured in RPMI media containing 20% FBS. B: The cells exposed to 25 µM diosgenin. C: H9C2 cells exposed to 100µM diosgenin

The IC_{50} (median growth-inhibitory concentration) determined by the MTT assay, was about 80 µM (Figure 2).

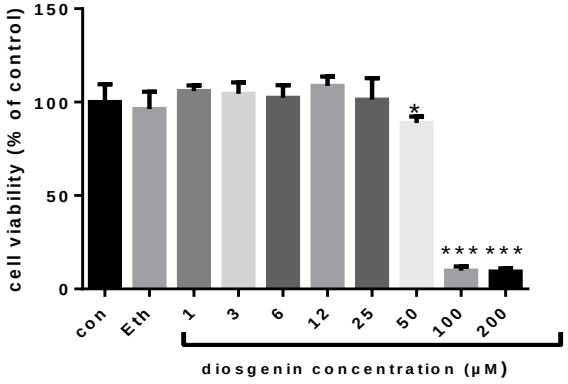

Figure 2. Cytotoxic Effect of diosgenin in H9C2 cells. Cells were treated with different concentrations of diosgenin for 24 h. The ratios of cell viability were measured by MTT assay. Data are presented as mean ± SEM of six replicates from three independent experiments. * p < 0.05 and ***p<0.001, compared to control

Protective Effect of Diosgenin on H9C2 Cells Against H_2O_2-Induced Cytotoxicity

The viability of H9C2 cells, which was measured by MTT method, decreased significantly (p<0.05) to 23.5% of the control values after cells were exposed to 200 µM H2O2 for 1 h (Figure 3).

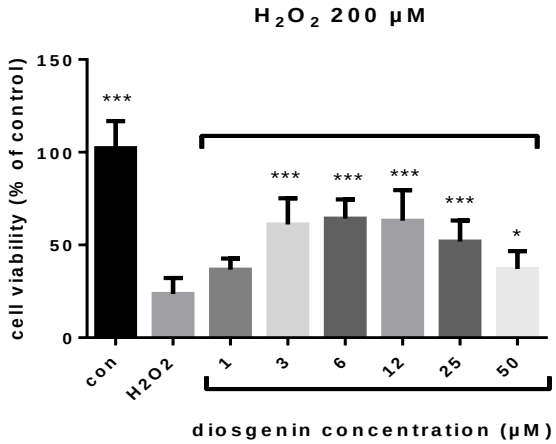

Figure 3. Diosgenin (1-50 µM) attenuated H9C2 cell loss mediated by 200 µM H2O2. Data are expressed as mean ±SEM. *p<0.05 and ***p<0.001, compared with H_2O_2 group

Pre-treatment with various concentrations (1 µM to 50 µM) of diosgenin for 24 h, significantly (p<0.05) increased cell viability (Figure 3). According to the results, microscopic images showed clearly an increase in the number of H9C2 cells after pre-treatment with diosgenin compared with cells treated with 200 µM H2O2 (Figure 4).

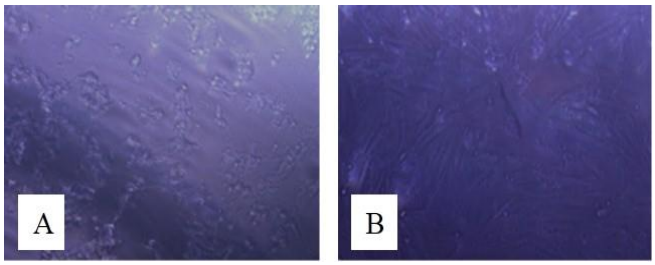

Figure 4. Morphological changes of H9C2 cells exposed to H2O2. Microscopic analysis of H9C2 cells after 1 h of treatment with 200 µM H2O2 in the presence of diosgenin. . A: H9C2 cells were incubated with 200 µM H2O2 for 1 h. B: The cells exposed to H2O2 200µM + Diosgenin 25µM.

Diosgenin Effect on The Reduction of Oxidative Stress

According to the above results, exposure of cells to H2O2 for 1 h (200 µM) caused a significant

increase in intracellular ROS generation. Pre-treatment of cells with different concentrations of diosgenin (3-25 µM) reversed this increase significantly ($p<0.001$) (Figure5).

In order to remove the effect of solvent, a group of cells were treated with ethanol alone. H2O2 increased the number of apoptotic cells and induced changes in the cell cycle phases.

In addition, treatment with diosgenin inhibited the effect of H2O2 on cell cycle phases and apoptosis. Our results show that diosgenin suppresses H2O2-indued cytotoxicity in H9C2 cells.

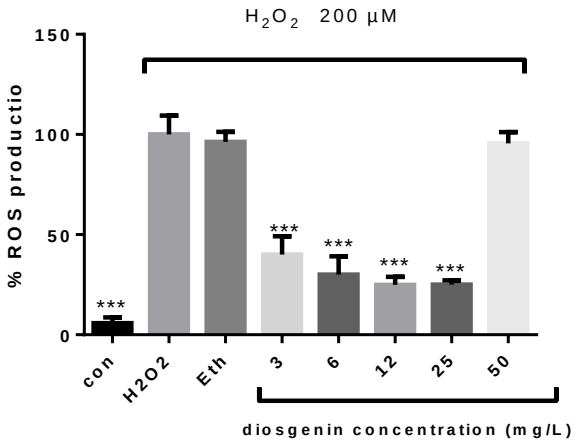

Figure 5. ROS production mediated by 200 µM H_2O_2 was reduced in diosgenin (3-25 µM) treated H9C2 cells. Data are expressed as the means±SEM of three independent experiments; ***p<0.001 compared with H_2O_2 group

Effects of Diosgenin on Cell Membrane Peroxidation

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation.

The treatment of H9C2 cells with 200 μM H2O2 during 1 h induced a significant increase of about 100% in the cellular concentration of MDA, indicating oxidative damage to the lipid content of cells.

On the contrary, pretreatment of H9C2 cells with 3-50 µM of diosgenin for 24 h prevented the MDA increase induced by H2O2, indicating a reduced level of lipid peroxidation in response to H2O2 (Figure 6).

Effects of diosgenin on SOD activity

The results showed that pretreatment of cells with 3-25 µM of diosgenin for 24 h reduced the SOD activity compare to H2O2 treated cells (Figure 7).

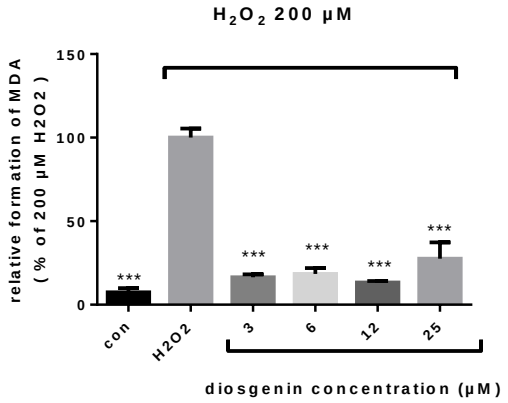

Figure 6. MDA production mediated by 200 µM H_2O_2 was reduced in diosgenin (3-25 µM) treated H9C2 cells. Data are expressed as means±SEM of three independent experiments; ***p<0.001 compared with H_2O_2 group

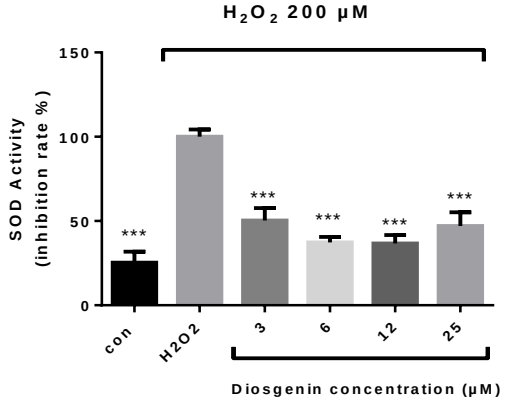

Figure 7. Diosgenin (3-25 µM) reduced SOD activity in H9C2 cells mediated by 200 µM H2O2. Data are expressed as means±SEM of three independent experiments; ***p<0.001 compared with H_2O_2 group.

Discussion

The effects of diosgenin on H9C2 cells were investigated in this study. In the first experiment, the toxicity of diosgenin on H9C2 cells was evaluated. Results clearly showed that diosgenin (50 µM) did not have any toxic effect on growth and proliferation of H9C2 cells. The IC_{50} determined by MTT assay was about 80 µM. Liu et al. (2005) reported that the IC_{50} of diosgenin on K562 cells was about 25 µM(Liu et al., 2005). Thus, Concentrations less than 80 µM were evaluated for protective effects on cell injury. The cells were treated with various concentration of diosgenin (1-50 µM) for 24h and then exposed to 200 µM H2O2 for 1h. The results revealed that pretreatment with various concentrations (1-25 µM) of diosgenin, significantly ($p<0.05$) increased cell viability. Consistent with these results, it was determined that increased cell

viability was due to reduced H2O2-induced oxidative stress by diosgenin. This is because diosgenin reduced the production of ROS to 25% compared with the cells only exposed to 200 µM H2O2 (control). Membrane lipid which contain unsaturated fatty acids, are particularly sensitive to oxidative stress, and their peroxidation leads to disturbance of the membrane integrity (Kaneko et al., 1990; Zaleska and Wilson, 1989). One important repair mechanism of damaged membranes is reacylation of phospholipids in the membrane (Zaleska and Wilson, 1989). In order to investigate the effect of diosgenin on peroxidation of membrane and rate of MDA production, the cells pretreated with diosgenin for 24 h, were exposed to H2O2. Diosgenin reduced peroxidation of membrane as well as the level of MDA especially at concentration 12 µM, especially. SOD activity in cells treated with diosgenin (3-25 µM) was significantly decreased compared with cells only exposed to H2O2.

Conclusion

The results of this study showed that treatment of cardiac H9C2 cells with diosgenin confers a significant protection against oxidative stress to the cells.

References

1. Accatino L., Pizarro M., Solis N. and Koenig C. S. (1998) Effects of diosgenin, a plant-derived steroid, on bile secretion and hepatocellular cholestasis induced by estrogens in the rat. Hepatology (Baltimore, Md.) 28:129-140.
2. Antunes F. and Cadenas E. (2001) Cellular titration of apoptosis with steady state concentrations of $H_{(2)}O_{(2)}$: submicromolar levels of $H_{(2)}O_{(2)}$ induce apoptosis through fenton chemistry independent of the cellular thiol state. Free Radical Biology and Medicine 30:1008-1018.
3. Aradhana, Rao A. R. and Kale R. K. (1992) Diosgenin--a growth stimulator of mammary gland of ovariectomized mouse. Indian Journal of Experimental Biology 30:367-370.
4. Attele A. S., Wu J. A. and Yuan C.-S. (1999) Ginseng pharmacology: Multiple constituents and multiple actions. Biochemical Pharmacology 58:1685-1693.
5. Beneytout J. L., Nappez C., Leboutet M. J. and Malinvaud G. (1995) A plant steroid, diosgenin, a new megakaryocytic differentiation inducer of HEL cells. Biochemical and Biophysical Research Communications 207:398-404.
6. Bernabucci U., Ronchi B., Lacetera N. and Nardone A. (2002) Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. Journal of Dairy Science 85:2173-2179.
7. Corbiere C., Liagre B., Bianchi A., Bordji K., Dauca M., Netter P. and Beneytout J. L. (2003) Different contribution of apoptosis to the antiproliferative effects of diosgenin and other plant steroids, hecogenin and tigogenin, on human 1547 osteosarcoma cells. International Journal of Oncology 22:899-905.
8. Davi G., Falco A. and Patrono C. (2005) Lipid peroxidation in diabetes mellitus. Antioxidants and Redox Signaling 7:256-268.
9. Kaneko M., Panagia V., Paolillo G., Majumder S., Ou C. and Dhalla N. S. (1990) Inhibition of cardiac phosphatidylethanolamine N-methylation by oxygen free radicals. Biochimica et Biophysica Acta 1021:33-38.
10. Kim D. S., Jeon B. K., Lee Y. E., Woo W. H. and Mun Y. J. (2012) Diosgenin induces apoptosis in HepG2 cells through generation of reactive oxygen species and mitochondrial pathway. Evidence-based complementary and alternative medicine: eCAM 2012:981675.
11. Liu M. J., Wang Z., Ju Y., Wong R. N. and Wu Q. Y. (2005) Diosgenin induces cell cycle arrest and apoptosis in human leukemia K562 cells with the disruption of Ca2+ homeostasis. Cancer Chemotherapy and Pharmacology 55:79-90.
12. Lores Arnaiz S., Travacio M., Llesuy S. and Rodriguez de Lores Arnaiz G. (1998) Regional vulnerability to oxidative stress in a model of experimental epilepsy. Neurochemical Research 23:1477-1483.
13. Moalic S., Liagre B., Corbiere C., Bianchi A., Dauca M., Bordji K. and Beneytout J. L. (2001) A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells. FEBS Lett 506:225-230.
14. P M. K., ey, Mittra P. and Maheshwari P. K. (2013) Oxidative stress in epilepsy with comorbid psychiatric illness. National Journal of Physiology, Pharmacy and

Pharmacology 3:92-96.
15. Peskin A. V. and Winterbourn C. C. (2000) A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clinica Chimica Acta; International Journal of Clinical Chemistry 293:157-166.
16. Pizarro J. G., Folch J., Vazquez De la Torre A., Verdaguer E., Junyent F., Jordan J., Pallas M. and Camins A. (2009) Oxidative stress-induced DNA damage and cell cycle regulation in B65 dopaminergic cell line. Free Radical Research 43:985-994.
17. Riley P. A. (1994) Free radicals in biology: oxidative stress and the effects of ionizing radiation. International Journal of Radiation Biology 65:27-33.
18. Roman I. D., Thewles A. and Coleman R. (1995) Fractionation of livers following diosgenin treatment to elevate biliary cholesterol. Biochimica et Biophysica Acta 1255:77-81.
19. Santanam N., Ramachandran S. and Parthasarathy S. (1998) Oxygen radicals, antioxidants, and lipid peroxidation. Seminars in Reproductive Endocrinology 16:275-280.
20. Srinivasan S., Koduru S., Kumar R., Venguswamy G., Kyprianou N. and Damodaran C. (2009) Diosgenin targets Akt-mediated prosurvival signaling in human breast cancer cells. International Journal of Cancer. Journal International du Cancer 125:961-967.
21. Stone J. R. and Yang S. (2006) Hydrogen peroxide: a signaling messenger. Antioxidants & Redox Signaling 8:243-270.
22. Thannickal V. J. and Fanburg B. L. (2000) Reactive oxygen species in cell signaling. American journal of physiology. Lung Cellular and Molecular Physiology 279:L1005-1028.
23. Toyokuni S. and Akatsuka S. (2007) Pathological investigation of oxidative stress in the post-genomic era. Pathology International 57:461-473.
24. Trevisan M., Browne R., Ram M., Muti P., Freudenheim J., Carosella A. M. and Armstrong D. (2001) Correlates of markers of oxidative status in the general population. American Journal of Epidemiology 154:348-356.
25. Turchan J., Pocernich C. B., Gairola C., Chauhan A., Schifitto G., Butterfield D. A., Buch S., Narayan O., Sinai A., Geiger J., Berger J. R., Elford H. and Nath A. (2003) Oxidative stress in HIV demented patients and protection ex vivo with novel antioxidants. Neurology 60:307-314.
26. Uchida K., Shiraishi M., Naito Y., Torii Y., Nakamura Y. and Osawa T. (1999) Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. The Journal of Biological Chemistry 274:2234-2242.
27. Ueda Y., Yokoyama H., Ohya-Nishiguchi H. and Kamada H. (1998) ESR spectroscopy for analysis of hippocampal elimination of a nitroxide radical during kainic acid-induced seizure in rats. Magnetic Resonance in Medicine: 40:491-493.
28. Wang S. L., Cai B., Cui C. B., Liu H. W., Wu C. F. and Yao X. S. (2004) Diosgenin-3-O-alpha-L-rhamnopyranosyl-(1 --> 4)-beta-D-glucopyranoside obtained as a new anticancer agent from Dioscorea futschauensis induces apoptosis on human colon carcinoma HCT-15 cells via mitochondria-controlled apoptotic pathway. Journal of Asian Natural Products Research 6:115-125.
29. Wei M. C., Zong W. X., Cheng E. H., Lindsten T., Panoutsakopoulou V., Ross A. J., Roth K. A., MacGregor G. R., Thompson C. B. and Korsmeyer S. J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science (New York, N.Y.) 292:727-730.
30. Yagi K. (1987) Lipid peroxides and human diseases. Chemistry and Physics of Lipids 45:337-351.
31. Zaleska M. M. and Wilson D. F. (1989) Lipid Hydroperoxides Inhibit Reacylation of Phospholipids in Neuronal Membranes. Journal of Neurochemistry 52:255-260.

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Research Article

Unravelling Over-Represented Amino Acids in Protein Structure of Allergen Proteins; a Large-Scale Study

Nassim Rahmani[1], Esmaeil Ebrahimie[1,2,3,4*], Ali Niazi[1], Najaf Allahyari Fard[5], Bijan Bambai[5], Zarrin Minuchehr[5], Mansour Ebrahimi[6]

[1] *Institute of Biotechnology, Shiraz University, Shiraz, Iran*
[2] *Division of Information Technology, Engineering and the Environment, School of Information Technology and Mathematical Sciences University of South Australia, Adelaide, Australia*
[3] *School of Biological Sciences, Faculty of Science and Engineering, Flinders University, Adelaide, Australia*
[4] *Schools of Medicine, The University of Adelaide, Adelaide, Australia*
[5] *National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran*
[6] *Department of Biology and Bioinformatics Research Group, University of Qom, Qom, Iran*

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Abstract

Allergens are proteins or glycoproteins which make widespread disorders that can lead to a systemic anaphylactic shock and even death within a short period of time. Understanding the protein features that are involved in allergenicity is important in developing future treatments as well as engineering proteins in genetic transformation projects. A big dataset of 1439 protein features from 761 plant allergens and 7815 non-allergen proteins was constructed. Thereafter, 10 different attribute weighting algorithms were utilized to find the key characteristics differentiating allergens and non-allergen proteins. The frequency of Leu, Arg and Gln selected by different attribute weighting algorithms with more than 50% confidence, including attribute weighting by Weight_Info Gain, Weight Chi Squared, Weight_Gini Index and Weight Relief. High amount of Gln and low percentage of Leu and Arg discriminate plant allergens from non-allergens

***Keywords*:** Plant allergens, Attribute weighting algorithms, Amino acid

Introduction

Allergy is an over-reaction of the immune system stimulated by allergens (Taylor and Hefle, 2006). Allergic reactions occur when allergenic proteins are detected by the antibody immunoglobulin E (IgE) (Van Gasse et al., 2015). During contact with the allergens, the immune system of allergic patients shows hypersensitive reactions (Ross and Montoya, 2015). Most plant allergens belong to only 10 proteins families such as Bi-functional inhibitor/lipid-transfer protein/seed storage 2S albumin, Profilin-like, Cupin, Bet V-1 like and etc. (our recent research- Unpublished). It indicated that conserved structure and biological activities play a role in determining and promoting allergenic properties (Breiteneder and Mills, 2005). Therefore, with finding the special characteristics of allergens the solution for reducing allergen properties could be offered. Investigating amino acid composition of nine different pollen allergens showed that the frequency of some amino acids such as Proline and Aspartic Acid were more than Arginine and Leucine (Mondal et al., 1998). Ara h 2, a peanut 2S albumin, has tough allergic reactions, but a homologous protein, soybean 2S albumin, is not introduced as an important allergen. Study of Structural difference between these proteins determined that some amino acids with a large side chain such as glutamine, and tyrosine were highly recognizable by the immune system (Youngshin et al., 2016).

Due to exponential increase in bioinformatics tools and techniques, huge amount of information from protein sequences can be obtained by computational biology offering a new vista for protein modeling such as various supervised (decision tree and neural network) and Unsupervised (with using the operators like K-Means, K-Medoids, Support Vector Clustering (SVC) and Expectation Maximization (EM)) machine learning algorithms and attribute weighting approaches. Weighting algorithm is a very simple method to determine important characteristics in a large database, by saving the time. According to weighted attributes we have

Corresponding authors E-mail:
* esmaeil.ebrahimie@adelaide.edu.au

better prediction and better decision. The benefit of attribute weighting for allergens is the fastest and easiest reactions to the new protein. Nowadays Attribute weighting algorithms are used in many researches to obtain original knowledge about the investigated traits such as influenza A virus (Ebrahimi et al., 2014), thermostable enzymes (Ebrahimi et al., 2011) and α-linolenic acid (ALA) (Zinati et al., 2014).

The aim of the present study was determined as amino acids frequency role in plant allergens and non-allergen proteins and find which amino acids lead the protein to be an allergen.

Materials and Methods

The structural protein attributes based on allergen and non-allergen proteins Sequence were extracted. Two Databases were created, one for plant allergens, next for Non- allergen proteins.

Data Collection

The plant allergens were collected individually according to the latest statistics data in allergen databases (SDAP, Allergenonlin, Allergome, and ADFS).

Data Pooling

A comparison of the information in different allergen sequences was performed. The information contained in the databases did not have the same formats as some of the allergens listed in some databases. All plant allergens collected in the primary secondary database were 2,424.

Data Cleaning

All plant allergens were cleaned and duplicated, while redundant and incomplete data were excluded using Clustal Omega, BioEdite and CD-HIT softwares. Finally, a total of 761 plant allergens remained.

Plant Allergens Database Creation

With more researches, attempts were made to prepare some common complete characteristics and features for all plant allergens data and eventually made our secondary database for plant allergens.

Non- allergen proteins Database Creation

Non- allergen proteins were collected randomly with omitting the isoforms, in uniprot database.

Protein Feature Extraction

The protein sequences were submitted in PROFEAT (Protein Features) webserver. It computed features from amino acid sequences and scored each attribute. PROFEAT is a web server for computing structural and physicochemical features of proteins and peptides from amino acid sequences and determine more than 1400 features for one protein. Eventually we performed a large scale functional analysis of 20 Amino Acid Composition between 8576 proteins (plant allergens and non-allergens).

Attribute Weighting

Ten different attribute weighting algorithms such as weighting by PCA, weighting by SVM, weighting by Relief, weighting by Uncertainty, weighting by Gini index, weighting by Chi Squared, weighting by Deviation, weighting by Rule, weighting by Information Gain Ratio, and weighting by Information Gain were used to find the amino acid composition role in separating plant allergens and non-allergen proteins. Attribute weighting algorithms find the characteristics which differ in protein structure between plant allergen proteins and the non-allergen proteins. The protein attributes which were assumed to be important by most attribute weighting algorithms (intersection of different weighting methods) were assumed as the key distinguishing features of allergen proteins from the non-allergen proteins.

Weighting by PCA (Principal Component Analysis)

This operator creates attribute weights of the Example Set by using a component created by the PCA. This operator behaves exactly the same way as if a PCA model is given to the Weight by Component Model operator.

Weighting by SVM (Support Vector Machine)

This operator calculates the relevance of the attributes by computing for each attribute of the input Example Set based on the weight with respect to the class attribute. The coefficients of a hyperplane calculated by an SVM are set as attribute weights.

Weighting by Relief

This operator calculates the relevance of the attributes by Relief. The key idea of Relief is to estimate the quality of features according to how well their values distinguish between the instances of the same and different classes that are close to each other.

Weighting by Uncertainty

This operator calculates the relevance of attributes

of the given Example Set by measuring the symmetrical uncertainty with respect to the class.

Weighting by Gini Index

This operator calculates the relevance of the attributes of the given Example Set based on the Gini impurity index.

Weighting by Chi Squared

This operator calculates the relevance of the attributes by computing for each attribute of the input Example Set based on the value of the chi-squared statistic with respect to the class attribute.

Weighting by Deviation

This operator calculates the relevance of attributes of the given Example Set based on the (normalized) standard deviation of the attributes.

Weighting by Rule: This operator calculates the relevance of the attributes of the given Example Set by constructing a single rule for each attribute and calculating the errors.

Weighting by Information Gain Ratio

This operator calculates the relevance of the attributes based on the information gain ratio and assigns weights to them accordingly.

Weighting by Information Gain

This operator calculates the relevance of the attributes based on information gain and assigns weights to them accordingly.

Results

Data were normalized before running the models; consequently all weights, regardless of the employed model, were between 0 and 1. The most important amino acids that were confirmed by 10 different weighting models to be involved in differentiation of plant allergens and non-allergen proteins are shown in Table 1. In this table, each weight shows the importance of each attribute regarding the target label based on its attribute weighting model. If one amino acid composition received weight higher than 0.5 (>0.5) by a certain attribute weighting algorithm, it was assumed to be an important amino acid supplement as shown in Table 1.

Weighting by PCA

In this model all attributes weighed a value of 0.0.

Weighting by SVM

Amino Acid Composition (%) L weighted more than 0.6 by this model.

Table 1. The most important Amino Acid Composition selected by different attribute weighting algorithms in discriminating allergen proteins from non-allergen proteins.

Amino Acid Composition	The number of attribute weighting algorithms that indicated the attribute as important
Leucine	5
Arginine	4
Glutamine	4

This table presents the number of algorithms that selected the attribute. Weighting algorithms were respectively PCA, SVM, Relief, Uncertainty, Gini index, Chi Squared, Deviation, Rule, Information Gain Ratio, and Information Gain.

Weighting by Relief

When this model is applied to the dataset, 8 Amino Acid Composition (%) showed weights higher than 0.5. The Amino Acid Composition (%) L and R both weighed value of 0.9.

Weighting by Uncertainty

No attributes resulted in weights higher than 0.5.

Weighting by Gini index

Again Amino Acid Composition (%) L, R and Q were weighted higher than 0.5.

Weighting by Chi Squared

5 Amino Acid Composition (%) were weighted higher than 0.5. The Amino Acid Composition (%) L and Q were selected by this model with weight of 0.9. The Amino Acid Composition (%) R weighed the highest possible weights of 1.0.

Weighting by Deviation

In this model all attributes weighed a value of 0.0.

Weighting by Rule

No attribute weighted more than 0.6 by this model.

Weighting by Information Gain Ratio

When this algorithm was applied to the dataset, 3 Amino Acid Composition (%) had weights higher than 0.5. and The Amino Acid Composition (%) Q weighed the highest possible weights of 1.0.

Weighting by Information Gain: In this model 4 Amino Acid Composition (%) weighed higher than 0.5. The Amino Acid Composition (%) L and R both weighed a value of 1.0. The result of 10 attribute weighting models to define important Amino Acid Composition (%) were different as shown in Table 2.

Table 2. The different algorithms weights for 20 Amino Acid Composition (%) in plant allergens and non-allergen Proteins

Weighting algorithm / Amino Acid Composition	PCA	SVM	Relief	Uncertainty	Gini Index	Chi Squared	Deviation	Rule	Info Gain Ratio	Info Gain
Alanine	0.0	0.0	0.6	0.1	0.1	0.2	0.0	0.0	0.5	0.1
Leucine	0.0	0.6	0.9	0.4	0.6	0.9	0.0	0.0	0.1	1.0
Methionine	0.0	0.1	0.4	0.1	0.2	0.1	0.0	0.2	0.7	0.2
Asparagine	0.0	0.1	0.3	0.0	0.1	0.0	0.0	0.0	0.0	0.1
Proline	0.0	0.0	0.4	0.1	0.2	0.3	0.0	0.0	0.2	0.3
Glutamine	0.0	0.3	0.5	0.4	0.7	0.9	0.0	0.4	1.0	0.6
Arginine	0.0	0.2	0.9	0.4	0.8	1.0	0.0	0.0	0.2	1.0
Serine	0.0	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Threonine	0.0	0.3	0.3	0.0	0.1	0.1	0.0	0.0	0.0	0.1
Valine	0.0	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tryptophan	0.0	0.1	0.4	0.0	0.0	0.1	0.0	0.0	0.0	0.1
Cysteine	0.0	0.4	0.3	0.4	0.4	0.5	0.0	0.0	0.1	0.5
Tyrosine	0.0	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Aspartic Acid	0.0	0.0	0.6	0.1	0.2	0.2	0.0	0.0	0.2	0.2
Glutamic Acid	0.0	0.1	0.6	0.1	0.2	0.3	0.0	0.0	0.0	0.3
Phenylalanine	0.0	0.3	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Glycine	0.0	0.2	0.4	0.3	0.4	0.5	0.0	0.0	0.2	0.4
Histidine	0.0	0.4	0.3	0.1	0.2	0.1	0.0	0.0	0.0	0.2
Isoleucine	0.0	0.0	0.4	0.1	0.1	0.1	0.0	0.0	0.0	0.1
Lysine	0.0	0.1	0.5	0.0	0.1	0.1	0.0	0.0	0.1	0.2

This table presents the attribute weights resulted from different algorithms

Altogether, the number of Amino Acid Composition (%) that gained weights higher than 0.5 in each weighting model were as follows: PCA (0 attribute), SVM (1 attributes), Relief (8 attributes), Uncertainty (0 attributes), Gini index (3 attributes), Chi squared (5 attributes), Deviation (0 attribute), Rule (0 attributes), Info Gain ratio (3 attributes) and Info Gain (4 attributes). The most important Amino Acid Compositions (%) that were confirmed by different weighting algorithms between plant allergens and non-allergen proteins are shown in Table 1.Three Amino Acid Composition of L, R and Q were selected by some different attribute weighting models. The average of Amino Acid Composition of L in plant allergens was 7.3 while in non-allergen proteins was 9.6. The mean value of Amino Acid Composition of Q L in plant allergens was 6.3 but in non-allergen proteins was 3.8. The average of Amino Acid Composition of R in plant allergens was 3.8 whereas in non-allergen proteins was 5.4 as showed in figure 1.

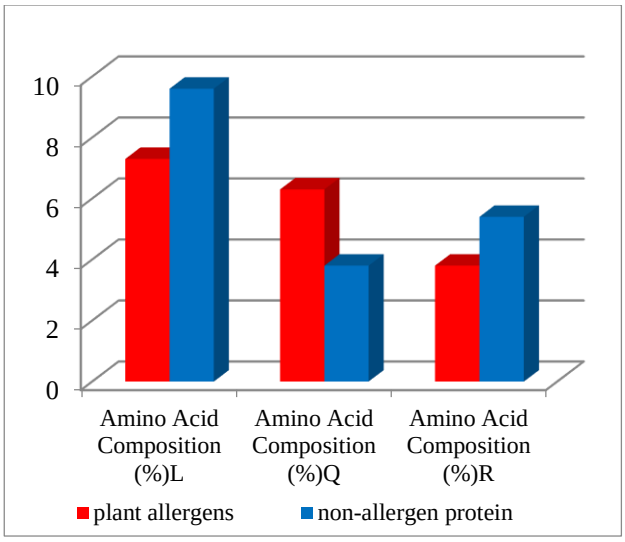

Figure 1. The mean value of three amino acids selected by attribute weighting algorithms in plant allergens and non-allergen proteins
1: Amino Acid Composition (%) L, 2: Amino Acid Composition (%) Q, 3: Amino Acid Composition (%) R

Discussion

The prevalence of allergic diseases and impact of allergic diseases are increasing worldwide. According to the World Health Organization, the number of patients having asthma is 300 million and with the rising trends it is expected to increase to 400 million, by 2025 (Pawankar et al., 2013). Thus any attempt to eliminate this problem is essential. Recently, a great attention has been paid to supervised machine learning methods implementing diverse amino acid composition and physic-chemical properties to unravel the underlying layers of protein function (Hosseinzadeh et al., 2012; Kumar et al., 2011). Mining of structural amino acid features have the potential to reflect these differences and help us to know specific changes which make a considerable impact on protein structure (Ebrahimi et al., 2014).

Each attribute weighting algorithm uses a specific pattern to define the most important features. Therefore, the results were different (Baumgartner et al., 2010; Bijanzadeh et al., 2010; Ebrahimi and Ebrahimie, 2010; Ebrahimi et al., 2011; Ebrahimie et al., 2011) as showed below: PCA, Relief, Gini Index, Chi Squared and Info Gain weighting models selected amino acid composition (%) L, with more than 50% confidence. Amino Acid Composition (%) Q Was selected by Relief, Gini Index, Chi Squared, Info Gain Ratio and Info Gain weighting algorithms with more than 50% confidence. Relief, Gini Index, Chi Squared, and Info Gain weighting models selected Amino Acid Composition (%) R, with more than 50% confidence.

Several research groups have studied and identified many different allergen proteins. Glutamine is one of the most abundant allergen protein. The studies reported that it has a great role in wheat allergens (Tanabe, 2001), peanut allergens (Youngshin et al., 2016), and also exists in pollen (Mondal et al., 1998). The Prolamin (an allergen superfamily) characterized by their high contents of glutamine (Shewry et al., 2002). This may explain why the frequency of Gln had higher weights in the applied attribute weighting algorithms we used in this study. Our results showed, more amount of Gln in plant allergens vs non-allergen proteins.

Study of homologous proteins, Ara h 2 allergen protein of Peanut with Soy Al 3 and Soy Al 1 of soybean, indicated a high level of glutamine in Ara h 2 epitopes in compared of corresponding areas in Soy Al 1 and Soy Al 3. So it can be concluded that may be glutamine is most recognizable by the immune system (Youngshin et al., 2016).

Our study demonstrated that the frequency of Leu was different in plant allergens and non-allergen proteins. The percentage of Leu in non-allergen proteins was more than plant allergens.

The investigation of 9 different allergen pollens composition of amino acids revealed an extremely low composition of leucine in only two of them (Mondal et al., 1998).

Our results determined the frequency of Arg was also weighted as a distinguishable effect on separating allergens from non-allergen proteins. The amount of this amino acid in plant allergens was lower than non-allergen proteins.

Evaluation of amino acids in pollen of 9 different allergen plants showed a small amount of arginine in only two of them (Mondal et al., 1998). Compere of Ara h 2, Soy Al 1 and Soy Al 3 revealed more percentage of Arg in some Ara h 2 epitopes, rather than Soy Al 1 and Soy Al 3(Youngshin et al., 2016), But the study had some limitations, by just focusing on Ara h 2, the result might not be enough to be generalized to other allergens also more than 30% of Ara h 2 epitopes doesn't have any Arginine in their sequence (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), and because of lacking in identification of epitopes that are associated with clinical reactions; the interaction, structure and composition study of antigen–antibody are needed.

Conclusion

The findings of this study indicate that the weighting models can be efficiently used for understanding the protein. According to the result of attribute weighting algorithms in a large-scale study, high amount of Gln and low percentage of Leu and Arg discriminate plant allergens from non-allergens.

Acknowledgments

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References

1. Baumgartner C., Lewis G. D., Netzer M., Pfeifer B. and Gerszten R. E. (2010) A new data mining approach for profiling and categorizing kinetic patterns of metabolic biomarkers after

myocardial injury. Bioinformatics 26:1745–1751.
2. Bijanzadeh E., Emam Y. and Ebrahimie E. (2010) Determining the most important features contributing to wheat grain yield using supervised feature selection mode. Australian Journal of Crop Science 4:402-407.
3. Breiteneder H. and Mills C. (2005) Plant food allergens—structural and functional aspects of allergenicity. Biotechnology Advances 23:395-399.
4. Ebrahimi M., Aghagolzadeh P., Shamabadi N., Tahmasebi A., Alsharifi M., Adelson D. L., Hemmatzadeh F. and Ebrahimie E. (2014) Understanding the underlying mechanism of HA subtyping in the level of physic-chemical characteristics of Protein. PLOS ONE 9.
5. Ebrahimi M. and Ebrahimie E. (2010) Sequence-based prediction of enzyme thermostability through bioinformatics algorithms. Current Bioinformatics 5.
6. Ebrahimi M., Lakizadeh A., AghaGolzadeh P., Ebrahimie E. and Ebrahimi M. (2011) Prediction of thermostability from amino acid attributes by combination of clustering with attribute weighting: a new vista in engineering enzymes. PLoS One 6.
7. Ebrahimie E., Ebrahimi M. and Sarvestani N. R. (2011) Protein attributes contribute to halo _ stability, bioinformatics approach. Saline Systems 7.
8. Hosseinzadeh F., Ebrahimi M., Goliaei B. and Shamabadi N. (2012) Classification of lung cancer tumors based on structural and physicochemical properties of proteins by bioinformatics models. PLoS One 7.
9. Kumar M., Gromiha M. M. and Raghava G. P. (2011) SVM based prediction of RNA binding proteins using binding residues and evolutionary information. Journal of Molecular Recognition 24:303-313.
10. Mondal A. K., Parui S. and Mandal S. (1998) Analysis of the free amino acid content in pollen of nine Asteraceae species of known allergenic activity. Annals of Agricultural and Environmental Medicine 5:17-20.
11. Pawankar R., Canonica G. W., Holgate S. T., Lockey R. F. and Blaiss M. (2013) The WAO White Book on Allergy.
12. Ross S. M. and Montoya M. I. (2015) Allergic reactions. *In* Basic Clinical Anesthesia. Springer. 197-202.
13. Shewry P. R., Beaudoin F., Jenkins J., Griffiths S. and Mills E. (2002) Plant protein families and their relationships to food allergy. Biochemical Society Transactions 30:906-910.
14. Tanabe S. (2001) Identification of wheat allergens. Internet Symposium on Food Allergens 3:163_170.
15. Taylor S. L. and Hefle S. L. (2006) Food allergen labeling in the USA and Europe. Current Opinion in Allergy and Clinical Immunology 6:186–190.
16. Van Gasse A., Mangodt E., Faber M., Sabato V., Bridts C. and Ebo D. (2015) Molecular allergy diagnosis: Status anno 2015. Clinica Chimica Acta 444:54-61.
17. Youngshin H., Jing L., Ludmilla B., Galina A. G., Chaeyoon L., Won H. S. and Hugh A. S. (2016) What characteristics confer proteins the ability to induce allergic responses? IgE epitope mapping and comparison of the structure of soybean 2S albumins and ara h 2. Molecules 21.
18. Zinati Z., Zamansani F., KayvanJoo A., Ebrahimi M., Ebrahimi M., Ebrahimie E. and MohammadiDehcheshmeh M. (2014) New layers in understanding and predicting α-linolenic acid content in plants using amino acid characteristics of omega-3 fatty acid desaturase. Computers in Biology and Medicine 54:14-23.

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Research Article

Construction of the Recombinant Lentiviral Vector Containing Human *GH1* Gene and its Expression in HEK293T Cells

Zahra Roudbari[1], Mohammadreza Nassiri[1, 2*], Mojtaba Tahmoorespur[1], Aliakbar Haddad-Mashadrizeh[2,3], Ali Javadmanesh[1]

[1] *Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Cell and Molecular Biotechnology Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*

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Abstract

Human growth hormone (*hGH*) is a protein with multiple roles in a range of biological functions such as protein, carbohydrates and lipid metabolisms as well as immunity, tissue development and overall growth. One of the major class of biopharmaceuticals in mammalian cells is the production of recombinant pharmaceutical proteins. In this study, we constructed a lentiviral vector carrying coding region of human GH1 (*hGH*) gene in order to production of recombinant *hGH* in mammalian cell line. *hGH* gene was amplified from a plasmid containing full-length *hGH* coding sequence and then cloned into the lentiviral vector pCDH-GFP. The HEK293T cells were transduced by the lentivirus particles as a targeted cell. *hGH* expression status in the recombinant cells were confirmed by RT-PCR. Additionally, western blotting analysis results showed that the recombinant cells maintained a stable *hGH* expression during five weeks of continuous culture. In conclusion, results of current study suggested that constructed lentiviral vector can potentially be used for a stable production of recombinant *hGH* protein in HEK293T cells. This methodology could be served as a foundation for further research and may open new insights toward therapeutic protein manufacturing.

***Keywords*: *hGH*, Recombinant lentivirus, Production protein, HEK 293 cells**

Introduction

There are large numbers of *in vitro* and *in vivo* studies proofing that *hGH* gene is a major regulator of growth (Levy, 2000). The growth hormone is a member of the somatotropin family (Yi et al., 2002) and currently, recombinant *hGH* has some therapeutic applications in the treatment of AIDS, dwarfism, bone fractures, skin burns and bleeding ulcers (Velloso, 2008).

Recombinant *hGH* is a protein that made to be very similar to the human growth hormone that is naturally active. This recombinant protein can enhance cell and tissue growth, linear growth (height), and metabolism of proteins, carbohydrates, lipids and minerals (Rezaei et al., 2012). Several expression systems have been established for recombinant protein production such as bacterial, yeast, insect and mammalian cells (Mao et al., 2015). Bacterial systems are the oldest and most extensively used expression systems, they are not appropriate to express eukaryotic proteins because of loss of correct post-translational modifications essential for full biological activity (Baneyx and Mujacic, 2004). Yeast expression systems often achieve higher yield than bacterial systems, and have the ability to express more complex proteins and conduct post-translational modifications necessary for complete biological activity (Daly and Hearn, 2005). Insect cell systems have become popular for recombinant protein production due to both short process development time and potential high yields. While, the glycosylation of insect cells is significantly different from mammalian cells because they are believed to be unable to process complex-type oligosaccharides (Kost et al., 2005). Expression systems based on mammalian cell lines for producing recombinant proteins have the capacity to perform different post-translational modifications and correct protein folding which are critical for biologically active proteins (Khan, 2013). Mammalian cells can express recombinant proteins through transient transfection and viral transduction (Gaillet et al., 2010). Transient expression systems

Corresponding authors E-mail:
* nassiryr@um.ac.ir

are not stable and may lead to loss of expression, therefore they are not efficient in addition to high costs of production. In contrast, lentivirus vectors have the ability to integrate into the nucleus of the target cells and provide stable expression of transgene in long term (Nayerossadat et al., 2012) when abundant amounts of material have to be generated on a routine basis (Lundstrom, 2003). This requires an expression system based on virus or on producing cell lines which have a stable integration of the inserted DNA in the host's genome. However, the integration of the recombinant constructs inside the host's genome, certainly result a wide range in protein synthesis levels within the same batch of cells (Assur et al., 2012). Considering the vast therapeutic applications of *hGH* and the approved successful application of lentivirus in transferring genes into mammalian cells, the goal of this study was, cloning the *hGH* gene into lentiviral vector and production of recombinant virus in HEK293T (human embryonic kidney) cells with the ability of expression of the *hGH* protein.

Materials and Methods

Cell Cultures

HEK293T cells (ATCC CRL-3216) (obtained from Mede Bioeconomy Company, Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen, USA) and incubated at 37C° in a humidified 5% CO_2 atmosphere.

Construction of hGH-Expressed Recombinant Lentivirus Vector

To amplify *hGH* sequence, a pair of primers (forward primer, 5´- **TCT AGA** ATG GCT ACA GGC TCC CGG -3´; reverse primer, 5´- **GCG GCC GC**C TAG AAG CCA CAG CTG CCC TC -3´) were designed and synthesize based on the *hGH* cDNA sequences (NM-000515.3). They had contained both *Xba*I and *Not*I restriction enzyme cutting sites (bold). The polymerase chain reaction (PCR) was used to amplify the *hGH* sequence using pUC57 plasmid contains *hGH* as a template. The thermal cycling condition was: 95 °C pre-denaturation for 5 min; 95 °C denaturation for 30 s, 56 °C annealing for 30 s, 72 °C extension for 30 s for 30 cycles; 72 °C extension for 7 min. The PCR products were electrophoresed in a 1% agarose gel, and ~550 bp *hGH* product was purified and cloned into the shuttle plasmid pCDH- GFP to construct the pCDH-*hGH*-GFP lentivirus vector. In order to confirm the clone of containing recombinant plasmid, enzymatic digestion was performed with *Xba*I and *Not*I enzymes and finally the recombinant pCDH- *hGH*- GFP plasmid was sequenced.

Production of hGH-expressed Recombinant Lentivirus Particles

The lentiviral vector were transfected with three plasmids pCDH- GFP, psPAX2 and pMD2.G (a gift from Tronolab) in HEK-293T cells, using the calcium phosphate method with some modifications (Trono, 2000; Roudbari et al., 2015). On day one, 5×106 HEK-293T cells were seeded in a 10 cm plate in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% FBS (Gibco, USA). On the second day, 21 µg of transfer vector, 21 µg of psPAX2 vector and 15 µg of pMD2.G vectors were mixed with transfection buffer and added drop-wised to the cells. Transfection medium was replaced with fresh medium within 14 hours post transfection. The expression of GFP was determined after 24 hours by fluorescent microscopy. The packaged recombinant lentiviruses were harvested from the supernatant of cell culture after 24, 48 and 72 hours post transfection. Then centrifuged at 2000 rpm at 4°C for 5 min and the supernatant filtered through a 0.22 µm filter. The recombinant virus was stored at -70°C for subsequent experiments. Viral titer was determined with counting the number of GFP positive cells by flow cytometry.

Transduction of HEK293T Cells

HEK293T cells at a concentration of 2×10^5 were seeded in a 6-well plate. The cells were transduced on the following day with recombinant lentivirus at a multiplicity of infection of 20. After 16 hours, transduction media was replaced with fresh DMEM containing 10% FBS. The transduced cells were passaged every three days. Transduced cells were assayed for GFP expression with a fluorescent microscope at 72 hours after infection and weekly for 5 weeks. Images were evaluated by Image J software (Jensen, 2013). GFP expression indirectly indicated expression of hGH and transduction efficiency of the recombinant lentivirus was assessed by flow cytometry.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

After 7 days of infection, total RNA was extracted from cells using RNeasy ® Plus Mini Kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized using QuantiTect Rev Transcription Kit (Qiagen, Germany), according to manufacturer instruction. RT-PCR conditions were as follows: a

cycle of 95°C for 5 min, and 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C 30 s and a cycle of 72°C for 7 min. The primer sequences were used to RT-PCR are listed in Table 1. PCR products were run on 1.5% agarose gel and stained with DNA safe stain (Sinaclone, Iran). Gel images were visualized with a UV transilluminator (SABZ biomedical, Iran) and the integrated optical density (IOD) of each band was measured by Image J sowftware. The levels of *hGH* mRNA were normalized against *β-actin*.

Table1. Primer sequences for RT-PCR.

Target gene	Primer sequences	Amplification length (bp)
***h*GH-F**	TAGAATGGCTACAG GCTCC	183
***h*GH-R**	GCTTCTTCAAACTCC TGGTAG	
β-actin-F	AGCCTCGCCTTTGCC GA	172
β-actin-R	CTGGTGCCTGGGGCG	

Western Blotting Analysis

From week-1 to week-5 after infection, the cells weekly were lysed in 200 µl of RIPA buffer (Thermo Fisher Scientific, USA) supplemented with protease inhibitor. The cell lysates were centrifuged at 11000 rpm for 12 min at 4ºC and the supernatants were collected. Protein concentrations of the supernatants were determined with a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Protein lysates (30 µg/lane) were loaded onto 12% SDS–PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, USA). The membranes were blocked with 5% non-fat dried milk and immunoblotting was performed with *hGH* (abcam, UK) and β-actin (Santa Cruz, USA) antibodies at 1:1000 dilutions. Anti-mouse IgG monoclonal antibody, conjugated with horseradish peroxidase at 1:2000 dilution. Finally the protein band was visualized by chemoluminescence reagent (ECL) and the integrated optical density (IOD) of each protein band was measured. IOD values were adjusted by internal standard β-actin. The concentration of recombinant *hGH* protein was determined by SDS-PAGE/Densitometry using ImageJ software.

Statistical Analysis

All experiments were conducted in triplicate and values were represented as mean ± SD. Statistical differences between groups were compared by one-way analysis of variance (ANOVA) using the SPSS 20.0 software. $P<0.05$ was considered statistically significant.

Results

Construction of Lentiviral Vector Containing *hGH* Gene

The coding sequence of *hGH* with 550 bp was successfully cloned into the pCDH- GFP vector and the recombinant lentiviral vector was named pCDH-hGH- GFP.

Sequencing results confirmed that the cloned *hGH* sequence was amplified and inserted correctly (data not shown). Digestion with *Xba* I and *Not* I enzymes also verified the accuracy of cloning (Figure 1).

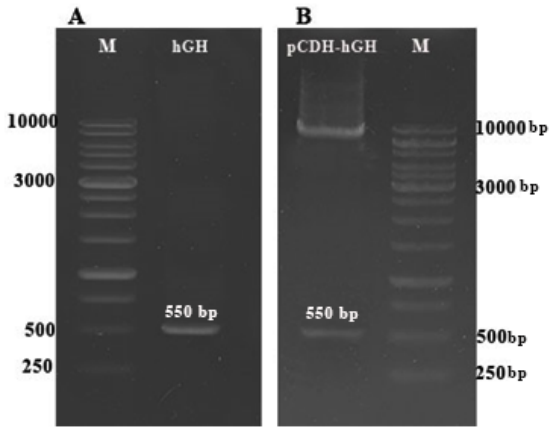

Figure 1. Construction of *hGH* recombinant lentiviral vector. A: PCR product of *hGH* and B: Recombinant pCDH-*hGH* digested by restriction enzymes *Xba* I and *Not* I, lane M: 1 kb DNA marker.

Packaging and Titration of The Recombinant Lentivirus Vector

The packaging of the recombinant lentivirus was verified using the expression of GFP by fluorescence microscope 24, 48 and 72 h after transfection. As shown in Figure 2, more than 90% of HEK293T cells were transduced by the lentivirus pCDH-hGH-GFP.

The lentivirus titer determination was based on the expression of GFP in a cell-based assay. The titer of the recombinant virus was approximately 1×10^7 IU/mL.

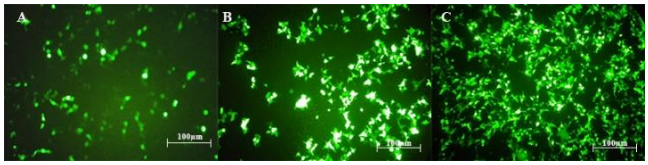

Figure 2. Fluorescent microscopy image showing GFP expression of HEK293T for achieving viral particles by pCDH- *hGH*. A: HEK 293T at 24 hours after transfection, **B**: HEK 293T at 48 hours after transfection and **C**: HEK 293T at 72 hours after transfection.

Recombinant Lentivirus Mediated GFP Expression in HEK293T Cells

Transduction efficiency of the recombinant lentivirus was initially investigated by analyzing GFP expression in HEK293T cells. The number of GFP-expressing cells and GFP intensity was assessed 3 days post transduction. Typically, at a multiplicity of infection of 20, more than 90% of the transduced cells were found to be GFP-positive using fluorescence-activated cell sorting analysis (Figure 3A and 3B). Therefore, HEK293T cells were successfully transduced with recombinant lentivirus with a high efficiency. Tracing transduced HEK293T cells with fluorescent microscope for 5 weeks showed that the percentage of GFP positive HEK293T cells did not change and GFP stably expressed (Figure 3C and 3D).

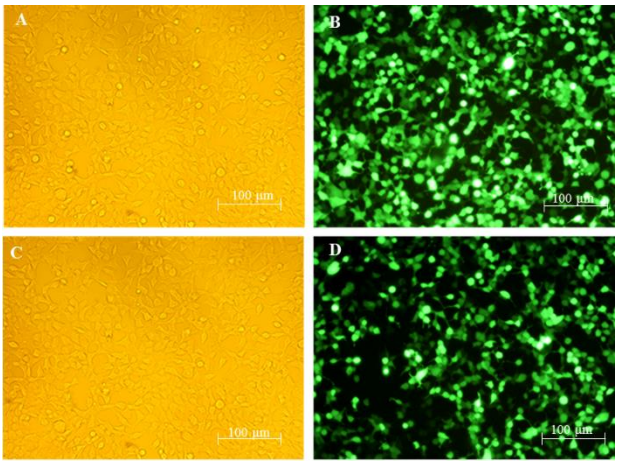

Figure 3. Transduction of HEK293T cells by recombinant lentivirus particles. A, B: Transduced HEK293T before and after fluorescent illumination; 72 h after transduction and C, D: Transducted HEK293T before and after fluorescent illumination; 5 weeks after transduction

The mRNA Expression of *hGH* in HEK293T Cells Using RT-PCR

The mRNA expression of *hGH* was measured using RT-PCR. The expression level of *hGH* gene was normalized to the expression of the β-actin gene, as reference gene. Based on the results of comparative analysis with Image J software, the transcription of *hGH* was detected in cells transduced with pCDH-*hGH* recombinant construct. There was no detectable transcript of *hGH* in HEK293T cell transduced by the pCDH-GFP (negative control) and non-transduced cells (Figure 4). The expression of *hGH* at mRNA level suggested that the pCDH-*hGH* lentivirus functionally integrated into the genome of HEK293T cells.

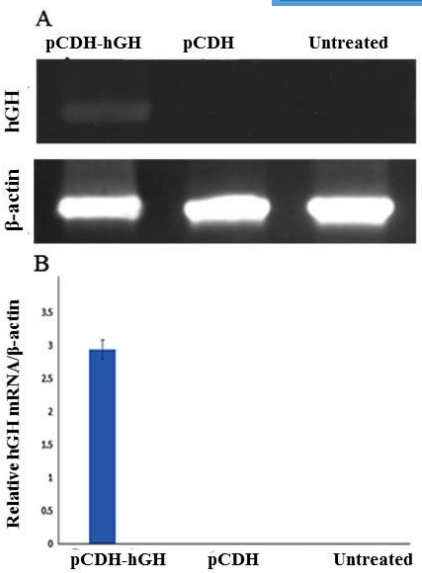

Figure 4. Effect of *hGH*-expressing recombinant lentivirus on the mRNA expression of *hGH* in HEK293T cells. A: RT-PCR analysis of different HEK293 cell groups after transduction. B: The *hGH* mRNA expression levels of different HEK293T cell groups were calculated using β-actin as an internal standard. (Data are means ±SD).

hGH Protein Expression in HEK293T Cells Using Western Blot

In order to confirm the expression of hGH protein in recombinant HEK293T cells western blot was performed. The result of immunoblotting analysis demonstrated that hGH protein was produced only in transduced HEK293T cells by hGH-expressed recombinant lentivirus (Figure 5).

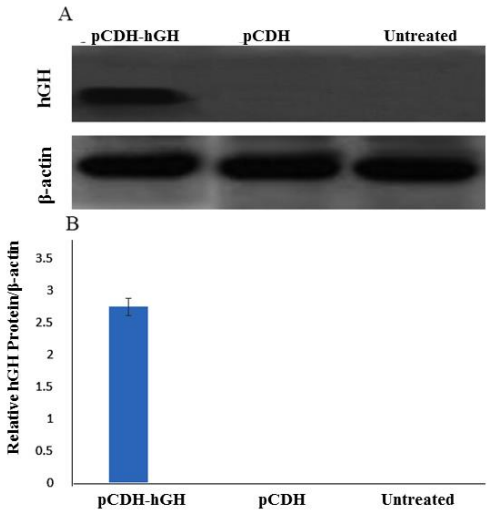

Figure 5. Effect of *hGH*-expressing recombinant lentivirus on the protein expression of *hGH* in HEK293T cells. A: Western blots analysis of different HEK293 cell groups. B: The *hGH* protein expression levels of different HEK293T cell groups were calculated using *β-actin* as a loading control. (Data are means ±SD).

Also results showed, recombinant HEK293T cell line maintained robust *hGH* expression for at least 5 weeks without any significant change in expression of gene (Figure 6). The concentration of recombinant hGH protein was calculated as 0.23 mg/ml by using densitometry analysis of band intensities from SDS polyacrylamide gel electrophoresis (SDS–PAGE) in Image J software.

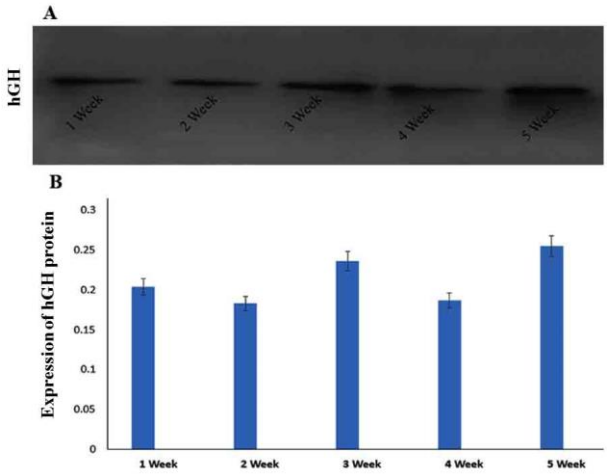

Figure 6. The sustained expression of *hGH* in transduced HEK293T cells by pCDH-*hGH* lentivirus. A: representative western blot showing sustained expressing of *hGH* from 1 week to 5 weeks. B: Bar graphs showing the expression of *hGH* levels in each time point. (Data are means ±SD).

Discussion

Recombinant pharmaceutical proteins are one of the most expensive and important therapeutic productions of the present time, and the production of recombinant proteins in mammalian cells represent a major class of biopharmaceuticals (Merlin et al., 2014).

Recent studies have demonstrated high efficiency for lentivirus transgenesis in mammalian cell lines including HEK293T and CHO (Huh et al.,2007). These cell lines can be widely used for production of recombinant proteins by viral-mediated transduction as well as by the formation of stable cell lines (Bell et al., 2015).

In the present study, we successfully constructed a lentiviral vector expressing *hGH* gene verified by restriction enzyme digestion and sequencing. This recombinant lentivirus had a bright form of green fluorescent protein (GFP): copGFP.

Numerous studies have shown that copGFP as a new version of GFP with boosted fluorescence that is more useful for enhanced visualization in vivo and in vitro (Day and Davidson , 2009; Alizadeh et al.,2015).

In current study, GFP was very functional in analysis of transfection efficiency in HEK 293T cells after packaging to produce recombinant viruses and assessment of transduction efficiency in transduced HEK293T cells. The results of fluorescent microscopy showed that HEK293T cells after transduction with recombinant lentiviruses expressed GFP stably during this experiment (5 weeks in this study).

It has been approved that compared with traditional non-viral shuttle vectors, Viral vectors have been widely used as shuttle vectors in recent studies due to their high efficiency in delivery, quick and long term effect and cost effective (MenezesK et al., 2006). Ma et al (2006) evaluated the efficiency of transfection of marrow stem cells with lentiviral vector and adenoviral vector, and they reported that the transfection efficiency of desired gene by lentivirus is considerably higher in compare to adenovirus.

As part of this study, we showed that the recombinant lentivirus could stably produce recombinant proteins in transducted cells after five weeks. In comparison with transient transfection, where production happened only in a short period (2-6 days) (Subramanian and Srienc, 1996), our results showed that the protein expression of hGH was stable for at least five weeks.

This fact was confirmed with the finding of Liu et al where they constructed a recombinant lentivirus vector mediated hGH in skeletal muscle myoblasts and results indicated long, efficient and stable expression of the recombinant hGH in skeletal muscle myoblasts (Liu et al., 2006). In another study, it was shown that the expression level of the desired protein by lentivirus was stable for at least 9 weeks (Mao et al., 2015).

The concentration of hGH protein in the current study showed that transduced HEK293T cells by recombinant lentivirus could approximately produce hGH with an increase of twice higher yield than E. coli which were transformed by plasmids containing hGH gene (Rezae and Zarkesh-Esfahani, 2012). In summary, the hGH-expressed by recombinant lentivirus evaluated in this study stably transduced HEK293T cells and resulted in long term expression. These findings provide valuable perception that could improve experiments leading to recombinant protein manufacturing especially in HEK 293T cells. The lentivirus expressing system is a highly efficient and also a simple gene transfer method. To our knowledge, this methodology could be served as a foundation for our further research and may be adapted for therapeutic protein manufacturing.

References

1. Levy R. M. (2000) Growth hormone therapy in adults and children. The New England Journal of Medicine 342(5):359.
2. Yi S., Bernat B., Pal G., Kossiakoff A. and Li, W.-H. (2002) Functional promiscuity of squirrel monkey growth hormone receptor toward both primate and nonprimate growth hormones. Molecular Biology and Evolution 19(7): 1083–1092.
3. Velloso C. P. (2008) Regulation of muscle mass by growth hormone and IGF-I. British Journal of Pharmacology 154(3):557-68.
4. Rezaei M. and Zarkesh-Esfahani S. H. (2012) Optimization of production of recombinant human growth hormone in Escherichia coli. Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences 17(7): 681–685.
5. Mao Y., Yan R., Li A., Zhang Y., Li, J., Du H. and Li H. (2015) Lentiviral vectors mediate long-term and high efficiency transgene expression in HEK 293T cells. International Journal of Medical Sciences 12(5):407–415.
6. Baneyx F. and Mujacic M. (2004) Recombinant protein folding and misfolding in Escherichia coli. Nature Biotechnology 22(11):1399–1408.
7. Daly R. and Hearn M. T. W. (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. Journal of Molecular Recognition 18(2): 119–138.8.
8. Kost T. A., Condreay J. P. and Jarvis D. L. (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nature Biotechnology 23(5):567–575.
9. Khan K. H. (2013). Gene expression in mammalian cells and its applications. Advanced Pharmaceutical Bulletin 3(2): 257–263.
10. Gaillet B., Gilbert R., Broussau S., Pilotte A., Malenfant F., Mullick A. and Massie B. (2010) High-level recombinant protein production in CHO cells using lentiviral vectors and the cumate gene-switch. Biotechnology and Bioengineering 106(2) : 203–215.
11. Nayerossadat N., Maedeh T. and Ali P. A. (2012) Viral and nonviral delivery systems for gene delivery. Advanced Biomedical Research 1:27.
12. Lundstrom K. (2003) Semliki Forest virus vectors for rapid and high-level expression of integral membrane proteins. Biochimica et Biophysica Acta 1610(1): 90–96.
13. Assur Z., Hendrickson W. A. and Mancia F. (2012) Tools for co-expressing multiple proteins in mammalian cells. Methods in Molecular Biology (Clifton, N.J.) 801: 173–187.
14. Trono D. (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene Therapy 7(1): 20–23.
15. Roudbari Z., Nassiri M. R., Tahmoorespur M. and Haddad-Mashadrizeh A. (2016) Lentiviral mediated overexpression of Insulin like Growth Factor-1 in mouse myoblast. Cellular and Molecular Biology 62(2): 111–115.
16. Jensen E. C. (2013) Quantitative analysis of histological staining and fluorescence using image J. The Anatomical Record 296(3):378–381.
17. Merlin M., Gecchele E., Capaldi S., Pezzotti M. and Linda A. (2014) Comparative evaluation of recombinant protein production in different biofactories: The green perspective. BioMed Research International (Article ID 136419) 14.
18. Huh S. H., Do H .J., Lim H.Y., Kim D.K., Choi S.J., Song H. and Kim J. H. (2007) Optimization of 25 kDa linear polyethylenimine for efficient gene delivery. Biologicals : Journal of the International Association of Biological Standardization 35(3);165–171.
19. Bell J., Parker K. L., Swinford R. D., Hoffman A. R., Maneatis T. and Lippe B. (2010) Long-term safety of recombinant human growth hormone in children. The Journal of Clinical Endocrinology and Metabolism 95(1);167–177.
20. Day RN. and Davidson MW. (2009) The fluorescent protein palette: tools for cellular imagining. Chemical Society Reviews 38(10); 2887-2921.
21. Alizadeh A., Soleimani M., Ai J., Fallah A., Hashemian S. J., Estiri H. and Nourani M. R. (2015) Lentiviral mediated overexpression of NGF in adipose-derived stem cells. Cloning and Transgenesis 4(3):4–8.
22. Menezes K. M., Mok H. S. and Barry M. A. (2006) Increased transduction of skeletal

muscle cells by fibroblast growth factor-modified adenoviral vectors. Human Gene Therapy 17(3): 314–320.

23. Ma X., Jiang J., Lu F., Ma X., Li X. and Huang H. (2006) Transduction of recombinant marrow stem cells using lentiviral vector and adenoviral vector: a comparative study. Chinese Medical Journal 86(47): 3340–3344.
24. Subramanian S. and Srienc F. (1996) Quantitative analysis of transient gene expression in mammalian cells using the green fluorescent protein. Journal of Biotechnology 49(1-3): 137–151.
25. Liu X.Y., Lu Y.X., Xu Y.L., Li X. Q., Liu J., Li A. H. and Wan J. P. (2006) Construction of human growth hormone lentiviral vector and its expression in murine skeletal myoblasts. Chinese Journal of Biotechnology, 22(2) : 243–248.
26. Mao Y., Yan R., Li A., Zhang Y., Li J., Du H. and Li H. (2015) Lentiviral vectors mediate long-term and high efficiency transgene expression in HEK 293T cells. International Journal of Medical Sciences 12(5): 407–415.

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Research Article

Early Non-invasive Determination of Fetal Sex Using Cell-free DNA

Sarreh Isakhani[1] and Ardeshir Bahmanimehr[2*]

[1] *Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran*
[2] *Thalassemia and Hemophilia Genetic, PND Research Center, Dastgheib Hospital, Shiraz University of Medical Science, Shiraz, Iran*

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Abstract

The first step in the prenatal diagnosis of X-linked genetic disorders is determining fetus gender. Current invasive methods to obtain the DNA source of the fetus instead of its miscarriage risk, has harmful stress for high risk pregnancies. Cell free fetal DNA (cffDNA) circulating in the maternal blood, has now become a useful source of noninvasive prenatal diagnosis. Considering limitation of cffDNA; as its small fragment size and low concentration in maternal plasma; using this source for clinical diagnostic material, requires a high efficiency extraction method and reasonable molecular tests to lead more accurate results.In the current study, we optimized Triton/Heat/Phenol (THP) protocol for extracting cffDNA in 8 and 12 weeks gestation. Fetal sex determined for prenatal diagnosis of hemophilia using SRY gene markers and high resolution markers of sex chromosomes by QF-PCR. The results compared with genetic tests on CVS samples. We confirmed the persistence of fetal DNA in maternal blood and investigated cell-free fetal DNA as a reliable approach in prenatal diagnosis of hemophilia. High accuracy and possibility of analyzing circulating fetal DNA in maternal blood highlights this method as a reliable one to early non-invasive determination of fetal sex to avoiding problems of invasive methods.

Keywords: Fetal DNA, Hemophilia, SRY gene, Prenatal diagnosis

Introduction

Particular Mendelian inheritance manner of X-linked diseases, accursed presence and expressing of phenotype in male form a mutation in a gene on the X chromosome.

Early diagnosis of genetic diseases linked to genes on the X chromosome is so important implications, as since those diseases only affect males. In most cases today, doctors diagnose X-linked diseases prenatally with invasive tests, such as chorionic villus sampling or amniocentesis, which carry a small risk of miscarriage. However, X-linked diseases cannot diagnose by fetal gender test, but it is possible to determine whether a fetus is male before conducting more invasive testing. The ability to determine the sex of a fetus accurately and early in pregnancy could reduce the number of invasive tests for sex-specific diseases by 50% (Costa et al., 2002).

In the routine classic clinical methods, for determination of fetal sex in the X-linked genetic disorders prenatal diagnosis, Duchenne muscular dystrophy (DMD) or hemophilia, Chorionic villus sampling (CVS) and amniocentesis should collected for genetic tests base on the extracted fetus DNA. A female fetus may have a wild-type genotype or be a carrier of X-linked genetic disorders, but further genetic analysis is crucial for a male fetus because a male fetus has a 50% change of having X-linked genetic disorders. Pregnant carriers risk miscarriage when undergoing such invasive prenatal diagnosis (IPD). Noninvasive prenatal diagnosis (NIPD) is preferable for fetal sex determination during the first trimester since it avoids the unnecessary risks of IPD in pregnant female X-linked genetic disorders carriers.

Cell-free circulating DNA (cffDNA) has been studied in a wide range of physiological and pathological conditions, including pregnancy, trauma, inflammatory disorders and malignancy (Costa et al., 2002; Pietropolli et al., 2016). It is present in normal healthy individuals at low concentrations (ng/ml) (Bianchi et al., 2001). For the first time, in 1997, Cell-free fetal DNA (cffDNA) was found in maternal plasma (Xue et al., 2009) and potential form of noninvasive prenatal diagnosis considered for it due to its measurement also its early

Corresponding authors E-mail:
**bahmani_a@sums.ac.ir*

detection, as early as 7 weeks, in maternal plasma. cffDNA comprises about 3-6% of the total cell-free DNA in maternal plasma (Wright et al., 2012).
The first trimester of pregnancy is clearly a critical period for prenatal diagnosis using non-invasive procedures (Yang et al., 2011). A technique for non-invasive fetal sex determination during the early trimester of pregnancy is Y chromosome (SRY) or other Y chromosome-specific sequences based on cffDNA from maternal plasma (Bianchi et al., 2001; Kimura et al., 2011; Wright et al., 2012; Xue et al., 2009; Yang et al., 2011).
To address the possibility that the investigation of cell-free fetal DNA can be a useful tool for the prenatal diagnosis of hemophilia by the assessment of Y-chromosomal sequences, we have analyzed maternal plasma for determination of the fetus gender.

Materials and Methods

Various methods have been used to purify cfDNA, including using modified salting-out, chromatography resins, magnetic beads, or guanidium thiocyanate (Keshavarz et al., 2015; Mackie et al., 2016; Qi et al., 2016; Swanson et al., 2013). The most popular is the QIAamp blood kit, which binds DNA to a silica-gel membrane, providing a fast and easy way to purify total DNA for polymerase chain reaction (PCR) analysis. Because DNA is present in plasma/serum at such Low concentrations (ng/ml), it is crucial to optimize laboratory protocols for the processing and extraction of cfDNA also comparing it by some standard commercial methods. In this study, we evaluated a simple Triton/Heat/ Phenol (THP) protocol and in parallel check its yield by the Bioscience cfDNA extraction kit.

Sampling and Extraction of cffDNA In Maternal Plasma

From patients referred to our center for PND test of hemophilia, some patients at about 9 weeks of gestation 8 mL blood samples were taken on the EDTA tube. The blood samples were centrifuged twice at 3,000 *g* and then at 12,000 *g* to obtain cell free plasma. Cell-free DNA was extracted from 2 mL of maternal plasma using Triton/Heat/Phenol protocol (THP) method. DNA was eluted into 40 µl of solution buffer. However, in some studies (Keshavarz et al., 2015; Mackie et al., 2016; Pietropolli et al., 2016), it recommended to separate plasma by double centrifugation at 800 *g* for 10 min, and 1600 *g* for 10 min and then immediately freeze it at -70°C.
For the THP method, 500 µl of plasma was mixed with 5 µl Triton X-100 (Sigma-Aldrich, UK) for better mixes, we incubate plasma and triton on 37 °C for 10 min and short vortex. After that, samples heat denatured at 98°C for 5 min and were placed on ice for 5 min to be cold. An equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v: v: v) (Sigma-Aldrich, UK) added to each tube, then centrifuged for 10 min at 14,000 g.
The aqueous upper phase was separated gently in the new tube to avoid mixing with down phase. 1/10 volume of 3 M NaOAc added to each tube within 2.5 volumes of 100% ethanol. The samples precipitated overnight with at -20°C.
Samples centrifuged for 30 min at 16000 *g* and removed supernatant. The DNA pellet was washed with 1000 µl ethanol 70% and following twice with 1000 µl absolute ethanol. Final washing with 100% ethanol should followed by 5 min centrifuge at 1600 *g* and remove carefully all aqueous phase. Samples air-dried and extracted DNA re suspended in 50 µl of ddH_2O. To optimize the protocol and determining if this DNA extraction method led to loss of small DNA fragments, the 100-bp-DNA Ladder was spiked into collected plasma. After re-purification, the ladder DNA was analyzed on 1.5% agarose gels with 0.5 µg/ml ethidium bromide.

Sex Determination Using SRY Gene Markers

The sequence of sex-determining region (SRY) markers of the Y chromosome was used to identify male fetal DNA present in each sample of a pregnant woman. To detect SRY gene on the samples we prefer Sequence-Tagged-Site (STS) Markers of SRY. STS marker in this region (SY14) under Gene bank access number G38356 is a short (469 bp) location known DNA sequence which is a single occurrence in the genome. For constructing genetic and physical maps of this STS marker we designed specific primers to detect it by the polymerase chain reaction (PCR).
Primer sequences were as follows:
SRY forward primer: GAATATTCCCGCTCTCCGGA
SRY reverse primer: GCTGGTGCTCCATTCTTGAG
Reactions were set up in a 20 µl volume using 50 ng of template, 1 µM of each primer, 100 µM of dNTPs and 1 unit of Taq Polymerase. The test consisted an initial 2 min at 92°C, a denaturation step of 95°C for 10 min, and then 30 cycles of 94°C for 1 minute, 58°C for 1.5 minutes and 72°C for 2 minutes. All samples were analyzed blindly with respect to fetal

gender. This method can indicate a male genotype by the presence of the amplified product from the SRY gene, but cannot accurately indicate a female genotype.

One of the reasons for this limitation is that the amplicon may be absent due to lack/degeneration of DNA in the examined specimen. The other reason is that a technical error during the examination process might cause a false negative result.

To solve this problem, also as complementary test, we used short tandem repeats (STRs) markers included in QF-PCR kits designed for testing chromosomal aneuploidies. In this test, homologous sequences in Amelogenin genes on the X and Y chromosomes (AMELX and AMELY) are simultaneously amplified and the sample of female origin can be discriminated from a false negative by single peak derived from the X chromosome on the electropherogram (Chan et al., 2004).

We used Aneufast QF-PCR kit to check high resolution markers of XY to assess sex determining markers also aneuploidy on X and Y chromosome for samples.

Results and Discussion

Optimization of THP Protocol

In the extraction of cffDNA, the obtained yield hardly depends on methods of sample collection, storage to different parts of the extraction protocol. Thus, optimizing the process is so important.

To optimize the yield of low-level cfDNA from plasma, we considered the effects of delays in blood processing and storage temperature prior to the DNA extraction also temperature of centrifuge and different mount of initial buffers for extraction. For each blood sample, different aliquots tests under different conditions of plasma collection and extraction protocol.

Finally THP protocol has optimized for our lab and used to experiment. The obtaining of cffDNA was differ in the different conditions, but there was no significant difference in cfDNA yield for samples processed up.

We tested different times also temperatures for incubation in the THP method and finally optimized, 98°C for denature plasma proteins also inactivate inhibitors of PCR. We used Triton X-100 for solubilization of protein instead of SDS to avoiding contamination of DNA.

For plasma collection method, we tested different time of delays in separating the plasma. Our finding was under contradicts of Jung et al., 2003 reports (Jung et al., 2003).

They reported that the DNA concentration in plasma did not change when blood samples were stored at room temperature for 8 hours, or store for 24 hours at 4°C.

We optimized the collected blood samples on the EDTA tubes and held for maximum 2 hours at room temperature or below before plasma separation. This method will be useful for avoiding leukocyte lysis and contamination of the plasma with genomic DNA.

Also for avoiding leukocyte lysis, we separated the Plasma from whole blood samples by separately double centrifugation for 10 minutes at 800 *g* and then in 1600 *g*.

After plasma preparation, we tested cffDNA extraction from frozen plasma on −70 °C and freeze plasma samples. However, there were no difference on cffDNA yield, but to avoiding DNA fragmentation in the repeated freezing and thaw cycles, we preferred start extraction immediately after plasma preparation. This finding is in the agreement of Xue, et al., 2009 and Keshavarz Z et al., 2015 reports on THP protocol.

To test the efficiency of extraction of small DNA fragments in the THP protocol, we added DNA ladder (100-bp) in the collected blood before cffDNA extraction.

1.5% agarose gel used to test extracted DNA and ladder fragments. The result showed the possibility of small fragments DNA extraction. However, for finding out the presence of DNA we prefer direct standard PCR for SRY marker and quantitative fluorescence PCR test for XY markers.

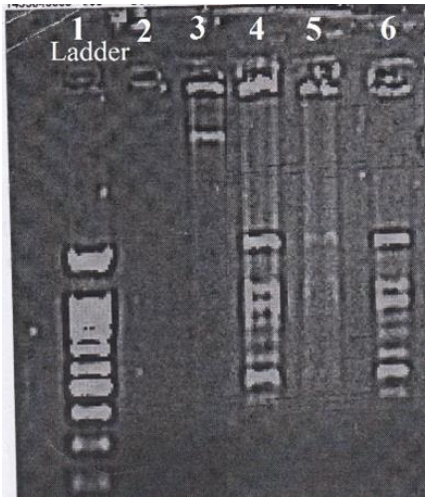

Figure 1. Detection of small DNA fragments in the THP protocol by adding ladder before extraction. 2 and 3: unsuccessful extraction sample 4,5 and 6 detected small fragments by extraction.

Research Article

Assessment of Male Fetal DNA Presence

An STS marker for the SRY region has been detected in normal male samples as positive control. It also checked in the female samples as negative sample. All extracted cffDNA tested for SRY presence as a male fetus. In different samples mount of DNA for this test optimized after the first test. We find out that gestation age has a direct effect on cffDNA yield. Gestation age 12 weeks yield more cffDNA comparing 8 weeks gestation. SRY negative samples did not lead clear result of female fetus due to the possibility of no DNA or degenerated DNA on the tested specimen.

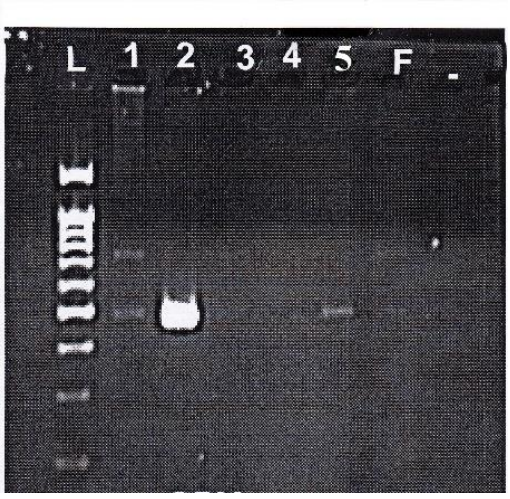

Figure 2. STS marker of SRY gene in some extracted cffDNA samples. 1 and 5: Male fetus. 3 and 4: Female fetus. 2: Control positive male sample F: Female sample as negative control.

For high accuracy of sex detection and eliminate the limitation of SRY test, all samples tested for 5 markers of sex chromosome. Figure 3 shows QF-PCR result of one of the samples. According to the kit manual, AMXY marker included two alleles with 104 and 110 bp size, which the last one belongs to the Y chromosome. The combination of this marker and SRY of the QF-PCR test is a common test to identify the sex of the sample. Which two alleles of AMXY and one allele of SRY indicates healthy X and Y chromosome in the fetus. SRY marker in healthy male should presence a 463 bp allele.

As a complementary test for aneuploidy of sex chromosomes we simultaneously tested X22, HPRT and DXYS218 marker indicating of a number of X and Y chromosome on the samples. X22 has two alleles on chromosome Xq28 and Yq by 226 and 229 bp size respectively. DXYS218 has 278 bp allele on Xp22.32 and 282bp allele on Yp11. HPRT has just allels on the Xq26.1 which number of the alleles indicate number of X chromosome on the sample. All studied markers in the sample of Fig.3 are two informative allele with a normal ratio of pick height and area except HPRT marker which has three allele. In this example, usually more high resolution markers on sex chromosomes should genotype to understand and distinguish the correct karyotype of the fetus.

Conclusion

For this study, we collected blood from 10 voluntary pregnant women in their 8 and 12 weeks of gestation age. They have applied for PND program for their hemophilia/thalassemia carrier mutations. For PND program, at the 12th week of gestation age CVS sample collecting to test the fetus genotype. We tested fetus gender from extracted cffDNA and CVS DNA in parallel for each sample. In the result we correctly detect 4 male and 6 female fetus, which results of both SRY and QF-PCR method had confirmed by CVS DNA tests.The results of this study showed that prediction accuracy of sex detection of SRY and QF-PCR method from cffDNA is high and would be useful in prenatal diagnosis as a pretest to detecting the fetus gender. Maternal plasma can use to extract cffDNA instead of unnecessary chorionic villus sampling on the pregnancies at risk of X-linked disease.

Acknowledgments

We are grateful to all patients who participate in the study by their blood samples and research permission on their CVS sample. Especial thanks to. Thalassemia and hemophilia Genetic, PND research center, Dastgheib hospital, Shiraz medical university of medical science for providing the samples.

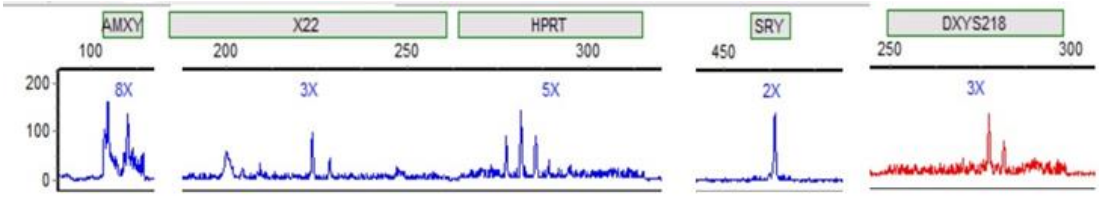

Figure 3. QF-PCR markers on sex chromosomes. A sample of extracted cffDNA, Male fetus.

References

1. Bianchi D. W., LeShane E. S. and Cowan J. M. (2001) Large amounts of cell-free fetal DNA are present in amniotic fluid. Clinical Chemistry 47:1867-1869.
2. Chan K. A., Zhang J., Hui A. B., Wong N., Lau T. K., Leung T. N., Lo K.-W., Huang D. W. and Lo Y. D. (2004) Size distributions of maternal and fetal DNA in maternal plasma. Clinical Chemistry 50:88-92.
3. Costa J.-M., Benachi A. and Gautier E. (2002) New strategy for prenatal diagnosis of X-linked disorders. New England Journal of Medicine 346:1502-1502.
4. Jung M., Klotzek S., Lewandowski M., Fleischhacker M. and Jung K. (2003) Changes in concentration of DNA in serum and plasma during storage of blood samples. Clinical Chemistry 49:1028-1029.
5. Keshavarz Z., Moezzi L., Ranjbaran R., Aboualizadeh F., Behzad-Behbahani A., Abdullahi M. and Sharifzadeh S. (2015) Evaluation of a modified DNA extraction method for isolation of cell-free fetal DNA from maternal serum. Avicenna Journal of Medical Biotechnology 7:85.
6. Kimura M., Hara M., Itakura A., Sato C., Ikebuchi K. and Ishihara O. (2011) Fragment size analysis of free fetal DNA in maternal plasma using Y-STR loci and SRY gene amplification. Nagoya Journal of Medical Science 73:129.
7. Mackie F., Hemming K., Allen S., Morris R. and Kilby M. (2016) The accuracy of cell-free fetal DNA-based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis. BJOG: An International Journal of Obstetrics & Gynaecology.
8. Pietropolli A., Capogna M. V., Cascella R., Germani C., Bruno V., Strafella C., Sarta S., Ticconi C., Marmo G. and Gallaro S. (2016) Three-hour analysis of non-invasive foetal sex determination: application of Plexor chemistry. Human Genomics 10:1.
9. Qi Q., Lu S., Zhou X., Yao F., Hao N., Yin G., Li W., Bai J., Li N. and Cram D. S. (2016) Copy number variation sequencing-based prenatal diagnosis using cell-free fetal DNA in amniotic fluid. Prenatal Diagnosis 36:576-583.
10. Swanson A., Sehnert A. J. and Bhatt S. (2013) Non-invasive prenatal testing: technologies, clinical assays and implementation strategies for women's healthcare practitioners. Current Genetic Medicine Reports 1:113-121.
11. Wright C. F., Wei Y., Higgins J. P. and Sagoo G. S. (2012) Non-invasive prenatal diagnostic test accuracy for fetal sex using cell-free DNA a review and meta-analysis. BMC Research Notes 5:1.
12. Xue X., Teare M. D., Holen I., Zhu Y. M. and Woll P. J. (2009) Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clinica Chimica Acta 404:100-104.
13. Yang Y.-H., Han S.-H. and Lee K.-R. (2011) Noninvasive prenatal diagnosis using cell-free fetal DNA in maternal plasma: clinical applications. Journal of Genetic Medicine 8:1-16.

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Research Article

Transient Expression of Coat Protein of Foot and Mouth Disease Virus (FMDV) in Alfalfa (*Medicago sativa*) by Agroinfiltration

Maziar Habibi-Pirkoohi[1*], Saeid Malekzadeh-Shafaroudi[1], Hasan Marashi[1], Saeid Zibaee[2], Afsaneh Mohkami[3], Saba Nejatizadeh[1]

[1] *Department of Biotechnology and plant breeding, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Razi Vaccine and Serum Research Institute, Ministry of Agriculture, Mashhad, Iran*
[3] *Research and Technology Institute of Plant production, Shahid Bahonar University, Kerman, Iran*

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Abstract

An *Agrobacterium*-mediated transient gene expression assay was carried out in alfalfa (*Medicago sativa*) leaves for expression of a chimeric gene encoding a part of capsid protein of Foot and Mouth Disease virus called VP1. The plant leaves were transformed via agroinfiltration procedure. The presence of the foreign gene and its expression in transformed plants were evaluated by polymerase chain reaction (PCR), real time PCR, protein Dot blot and ELISA. Moreover, gene expression in the transformed leaves was quantified by ELISA method. The results obtained in this investigation indicated high level of gene expression in alfalfa leaves, showing that transient gene expression can be applied as an effective and time-saving procedure for the production of recombinant proteins. The procedures for transformation, detection of recombinant protein and its application for molecular experiments are described in the study.

***Keywords*:** Agroinfiltration, FMDV, Recombinant vaccine, Alfalfa, VP1

Introduction

In recent years, green plants have been widely applied for the expression of foreign pharmaceutical proteins including recombinant vaccines; however, the long time required for producing transgenic plants together with the high cost and low protein yield are the major obstacles to commercialization of plant-based molecular farming (Wroblewski et al., 2005). An appropriate alternative for conventional genetic engineering procedures is application of transient gene expression using *Agrobacterium tumefaciens*. In this method, the suspension of *A. tumefaciens* containing the gene of interest is transferred to plant leaves either with a needle-free syringe or a vacuum infiltration and the expression of foreign genes on TDNA usually reaches to its maximum at 2−3 days post-infiltration (Habibi et al., 2014). Indeed, transient gene expression systems have been extensively used by many authors as a simple, cost-effective, fast and reliable method for a wide range of experiments including gene function (Sohn et al., 2011), protein production (Vaquero et al., 1999), host–pathogen interaction (Tang et al., 1996) protein–protein interaction (Bhat et al., 2006) and protein localization (Doran, 1999). So far many plant species have been used for the production of recombinant vaccine; the most notable examples being tobacco, potato, tomato, banana, corn, lupine and lettuce (Carter et al., 2002). Choosing the plant species for expression of recombinant vaccine is an important issue which is mainly determined by considering how the vaccine is going to be used. Edible plant species such as vegetables are appropriate candidates if the vaccine is planned for raw consumption (Sala et al., 2003). In the case of veterinary vaccines, forage crops would be the choice of interest (Walmsley et al., 2000).

Foot and Mouth Disease (FMD) is a highly contiguous animal disease with harmful effects on milk- and meat-producing animals (Wang et al., 2002). There have been many efforts to produce recombinant vaccines against this disease in plant systems (Habibi and Zibaee, 2013). However, to the best of our knowledge, no investigation on the production of FMD recombinant vaccine via transient gene expression in plant host has been reported. The capsid of Foot and Mouth Disease virus (FMDV) is composed of four structural polypeptides designated VP1, VP2, VP3 and VP4

Corresponding authors E-mail:
*Maziar.habibi@alumni.um.ac.ir

(Bachrach et al., 1975).
The prominent G–H loop of the VP1 capsid protein of FMDV, spanning residues 134–158, has been identified as the major immunogenic site for neutralizing antibodies (Rodriguez et al., 2009). Moreover, G-H loop flanking regions have been shown to boost its immunogenicity by inducing B cells and T-helper cells (Wang et al., 2002).
This paper reports the production of a novel recombinant vaccine against FMD in alfalfa leaves through *Agrobacterium*-mediated transient gene expression. The synthetic gene designed for this study included a DNA fragment encoding 129 to 169 amino acids of VP1 capsid protein. This involved both G-H loop and its flanking regions, so was expected to be an effective tool for inducing immune response in animal host. The gene construct was further elaborated by the inclusion of eukaryotic ribosome binding site (Kozak sequence) and an endoplasmic reticulum signal peptide (SEKDEL) as described in materials and methods section. Alfalfa was adopted as a host plant in this study since it is a forage crop constituting an ordinary portion of livestock diet. This makes alfalfa a suitable candidate for the production of recombinant vaccines against FMD.

Materials and Methods

Construction of synthetic VP1 gene

A 120 bp long fragment of VP1 encoding 129-169 amino acids of VP1 capsid protein was designed as the main part of expression construct. A eukaryotic ribosome binding site called Kozak sequence, GCCACC, was introduced prior to the start codon and an endoplasmic reticulum signal peptide called SEKDEL consisting of six amino acids was attached to 3′ end just before stop codon. Start codon (AUG) and stop codon (UAA) were also added into the 5' and 3' ends of the construct, respectively. Recognition sites of *Bam*HI and *Sac*I restriction enzymes were introduced into the 5' and 3' ends of the synthetic gene, respectively (Figure 1). The construct was synthesized and cloned into the pGem T-Easy vector (Bioneer, South Korea).

Construction of a Binary Plant Expression Vector

The synthetic VP1 gene fragment was digested from pGem T-Easy vector by *Bam*HI and *Sac*I and was inserted into the plant expression vector pBI121 downstream of the CaMV 35S promoter and upstream of the nopaline synthase (NOS) terminator, yielding pBI121-VP1vector. The ligation reaction mixture was used to transform *E. coli* strain DH5-α and kanamycin-resistant colonies were isolated after overnight incubation at 37°C. After bacterial growth, the plasmid was extracted from bacterial cells using alkaline lysis method. The plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by so called thaw-melting method. In summary, a suspension of bacterium with OD= 0.6 (600 nm) was placed on ice for 15 min. 1.5 ml of the suspension was centrifuged at 4000 *g* for 10 min. Supernatant was removed and 1 ml of cold $CaCl_2$ (20 mM) was added to bacterial pellet. The pellet was solved by vortex. 1 µgr of recombinant plasmid was added and mixed. The reaction tube was frozen in liquid nitrogen for 2 min and then placed at 37ºC for 5 min. One milliliter of LB medium was added and the solution was placed in shaker incubator at 28ºC. The suspension was again centrifuged; the supernatant was removed so that only 100 µl of the suspension along with the bacterial pellet remained in the tube. The pellet was mixed with the culture medium and spread on solid culture containing LB agar medium supplemented with 50 mg/l Kanamycin, 50 mg/l rifampicin and 20 mg/l gentamicin. The recombinant colonies appeared after 48 hours. The putative transformed cells were further evaluated by PCR assay.

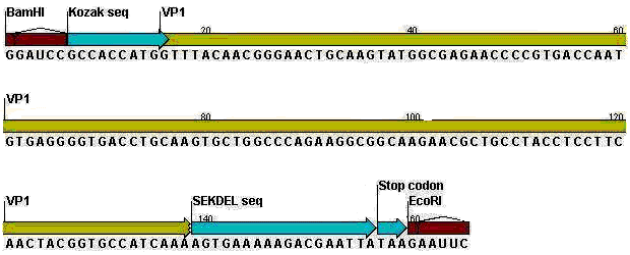

Figure1. Schematic presentation of the synthetic VP1 gene

Plant transformation

Single colony of *Agrobacterium* containing pBI121-VP1 plasmid was cultured for 48h on LB medium (NaCl 10 g/L, yeast extract 5 g/L, tryptone 5 g/L) supplemented with gentamicin 10 mg/l, rifampicin 50 mg/l and kanamycin 50 mg/L. after reaching density of OD_{600} = 1.5, the cultures were centrifuged, the supernatant was discarded and the pellet was resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 µM acetosyringone) and density was adjusted to OD_{600} = 0.5. The suspension was incubated for 2 h at room temperature. *Agroinfiltration* was then carried out to transform alfalfa leaves. The bacterial suspension was transferred to alfalfa leaves with a needle-free syringe as described by Sparkes et al (2006). Alfalfa plants were placed in growth chamber for three days

under 25°C, 16 h light/8 h darkness photoperiod and 75% humidity and then they were analyzed.

Detection of VP1 gene in transgenic plants

Detection of VP1 gene in transgenic lines and other molecular analyses were conducted three days after agroinfiltration. PCR analysis was performed to evaluate presence of the expression cassette in the leaf tissue of transformed alfalfa plants. Genomic DNA was extracted from leaves of transgenic plants using modified Dellaporta method and used as template for PCR analysis using specific primers. The sequence of forward and reverse primers were 5' ATGGAAATTGTAAGTATGGAGA 3' and 5' GAAGAAAGCGAAAGGAGC 3' respectively. The forward primer matches a sequence within VP1 and reverse primers matches NOS terminator. Genomic DNA of wild type plants was used as negative control. PCR was carried out by 30 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 45s, followed with a final extension step at 72°C for 10 min.

Real Time PCR assay

Real Time PCR assay was performed to analyze gene expression at transcription level. Total RNA was extracted from leaf tissue and complementary DNA (cDNA) was synthesized via reverse transcription using oligo(dT) 20 primer. The cDNA mixtures were used as templates for real-time PCR. Specific forward and reverse primers for Real Time PCR were 5' ATGGAAATTGTAAGTATGGAGA 3' and 5' ATTAAAAGAAGTTGGAAGAGTT 3', respectively. Data were normalized to the expression of Aspartate Aminotransferase (ATT), a housekeeping gene, which has stable expression under different experimental conditions in similar studies. Specific forward and reverse primers for ATT gene were 5'CAATTTCGCATCTCATTAAGATCG3' and 5'ACCACATCCCAAATAAATAAGATTCTAAC 3', respectively.

The efficiency of primer binding was determined by linear regression by plotting the cycle threshold value versus the log of the cDNA dilution.

Expression of the synthetic gene was quantitatively analyzed using a Real-Time PCR system (BioRad). Real-Time PCR was carried out in a 20 µL reaction volume containing 0.5µM of each primer and 10 µl of SYBR Green Real time PCR master mix (Genet Bio, South Korea). Quantitative Real-Time PCR experiments were performed in duplicate for each sample. Student's t-test was used to evaluate the statistical significance of the data; $p < 0.05$ was considered statistically significant.

Protein dot blot assay

Expression of VP1 gene in alfalfa leaves was evaluated using protein dot blot assay. Briefly, total protein was extracted using Tris-HCL method. Small samples of the protein (3 µl) were dotted on nitrocellulose membrane and allowed to dry. BSA (Bovine Serum Albumin) was used to prevent non-specific antibody reactions. The membrane was then incubated for 60 min at 37°C with primary antibody (1:2000 dilution), washed three times with PBS (Phosphate Buffer Saline) and PBST and finally incubated with secondary conjugated antibody (1:1500). Color was developed by adding OPD (Ortho-Phenylenediamine). Protein sample of non-transformed plant was used as negative control and a 3 µl of pure synthetic peptide corresponding to VP1-129-169 amino acids (Bioneer, South Korea) was used as positive control.

ELISA assay

Expression of the foreign gene was further evaluated using enzyme-linked immunosorbent assay (ELISA). ELISA plate was coated with total soluble proteins from the wild type and transformed plants and known FMDV VP1 antigen at 37°C for one hour; followed by incubation with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C to prevent non-specific binding. The well was washed with PBST/PBS, incubated with antiserum reactive against FMDV (1:1000 dilutions) and then alkaline phosphatase conjugated with anti rabbit IgG (1:1500). Wells were developed with TMB (Tetramethyl benzidine) substrate; the color reaction was stopped by 2 N H_2SO_4 and read at 405 nm of wavelength.

Quantification of the recombinant protein in transformed leaves

Total soluble protein concentration was determined using Bradford assay. For quantification of recombinant protein content in infiltrated leaves, standard curve for VP1 was drawn using known amounts of a synthetic VP1-129-169 peptide (Bioneer, Sout Korea). For this, serial dilutions of the synthetic VP1-129-169 peptide were used in ELISA assay and the absorbance of each well was measured.

Then, absorbance (Y axis) was plotted against protein concentrations (X axis). The curve was used to determine concentration of recombinant protein in transformed leaves (figure 6). Concentration of the recombinant VP1 protein was calculated by dividing VP1 concentration by total soluble protein, and expressed as the percentage of total soluble protein (%TSP); as follows:

%TSP = [*VP1* concentration/ total protein concentration] × 100

Results

Transgene detection in infiltrated leaves

The presence of expression cassette in *A. tumefaciens* and transformed plants was evaluated using PCR analysis. PCR products were separated on 1% agarose gel by electrophoresis. The 587bp band of foreign gene was observed in transgenic plant and *A. tumefaciens* colony. No band was amplified from non-transformed plant (Figure 2).

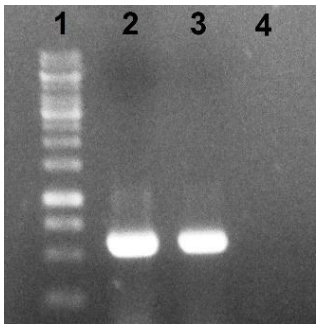

Figure2. PCR analysis for detection of VP1 gene in transformed leaves of alfalfa. 1) 1 kb ladder; 2) plasmid pBI121VP1 (positive control); 3) transformed alfalfa plant; 4) wild type plant (negative control)

Evaluation of transgene expression

Expression of the foreign gene was measured at transcription level using Real Time PCR. The results of Real Time PCR confirmed VP1 gene expression in all transformed samples but no signal was detected for control line (Figure 3). As can be inferred from Figure 3, transcription rate was quite high in transformed leaves. The difference between infiltrated leaves was not significant ($p<0.05$).

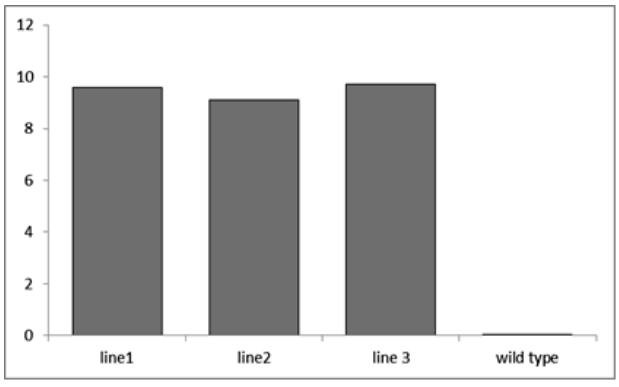

Figure 3. Quantitative measurement of VP1 gene transcription in transformed leaves of alfalfa via Real Time PCR. Data presented in this graph are obtained from three samples of transformed plants.

Expression of VP1 was further evaluated in translational level by dot blot and ELISA assays.

The production of recombinant VP1 protein was measured by dot blot assay. Positive signal showing specific antigen/antibody reaction was observed for protein samples obtained from transformed alfalfa plants and for those samples from positive control as well. As expected, no signal was detected for protein sample of non-transformed plant (Figure 4).

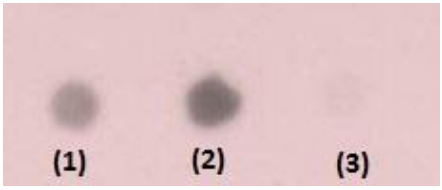

Figure4. Dot blot assay for detection of recombinant protein in transiently transformed leaves of alfalfa. (1): protein sample of transformed leaves, (2) pure VP1-129-169 peptide as positive control, (3): protein sample of wild type plant (negative control)

Expression of VP1 recombinant protein was quantitatively assessed using ELISA assay. ELISA results showed that the recombinant protein was produced in the samples obtained from the infiltrated leaves, whereas no detectable signal was observed for that of non-transformed plant (Figure 5).

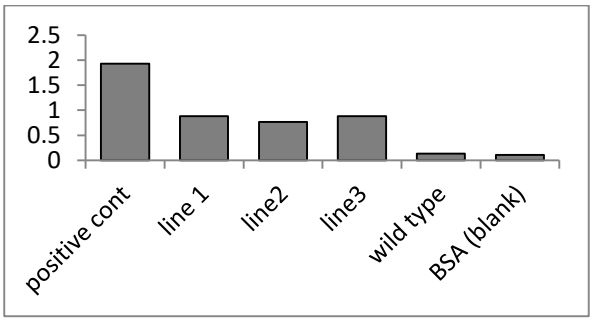

Figure5. Quantification of recombinant VP1 expression in three transgenic alfalfa plants by ELISA.

Finally, concentration of recombinant VP1 was quantified in the transformed leaves. Figure 6 shows the standard curve of VP1 protein. As determined by standard curve of Bradford, total protein concentration of the transformed leaves was 4.4 µg/ µl. Using standard curve of pure synthetic VP1 protein, concentration of recombinant protein was calculated as 0.042 µg/ µl; therefore final concentration of the recombinant VP1 protein was:

0.042/ 4.4 × 100= 0.95%TSP

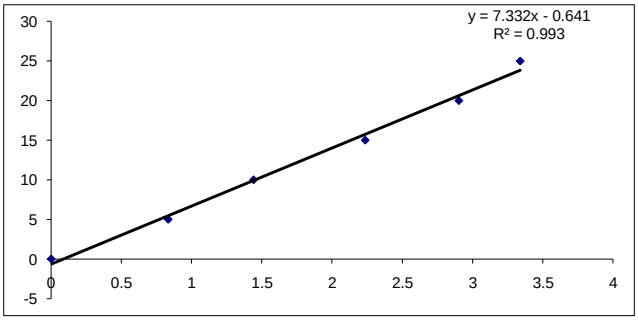

Figure 6. Standard curve of VP1 protein

Discussion

In the present study, alfalfa leaves were transiently transformed with a chimeric construct of VP1 gene via agroinfiltration method. The method has been reported as an efficient and rapid procedure for transient gene expression in plants (Sohn et al., 2011)

PCR assay confirmed presence of the synthetic construct in infiltrated leaves. In transient expression assays, the gene of interest is not integrated in nuclear genome of plant cell. Thus, copy number of the transgene in plant tissue is high and PCR product band in electrophoresis is almost as sharp as that of pure plasmid (Figure 2).

The results of this study demonstrated that agroinfiltration can be a fast and efficient tool for production of recombinant vaccines in intact plants. As confirmed by Real Time PCR assay, transient expression level of the transgene was fairly high which was in agreement with the results obtained by Leckie and Stewart (2011) who reported high level of gene expression in leaves of *Nicotiana benthamiana* through agroinfiltration. Indeed, some investigators have claimed that transgene expression level in transient expression assays can be up to 1000 fold higher than that of stable transformation (Sparks et al., 2006). Although such a high expression level was not observed in the present study, the expression of VP1 was of great magnitude (0.95%TSP) as quantified by dividing concentration of the recombinant protein by total soluble protein concentration. It is quite surprising that in spite of the wide range of experimental purposes of transient gene expression, there have been few studies on the application of this transformation approach for producing recombinant vaccines in plant systems. Most of the works in the field of transient antigen expression in plant hosts have been conducted by means of plant viruses as vehicle for gene delivery and expression, in which the epitope of interest is usually inserted within the coat protein gene (Koprowski et al., 2001). This method has proved to be an efficient and rapid way for the production of recombinant protein in plants but is limited by the fact that construction of viral vector for expression of foreign protein is much laborious and time-consuming. Moreover, when the size of foreign gene exceeds a certain threshold, efficiency of the viral vector is reduced (Sala et al., 2003). In contrary, genes with large size can be efficiently expressed in plants via *Agrobacterium*-mediated genetic transformation. In the other words, agroinfiltration (and other types of *Agrobacterium*-mediated transient gene expression) combines advantages of both viral-based transient gene expression, that is the production of recombinant protein in a short time, and *Agrobacterium* mediated transformation, the ability to transfer large foreign genes. This makes agroinfiltration a promising alternative for the production of recombinant vaccines in plant-based systems.

Plant choice is a critical issue in production of recombinant vaccines and other pharmaceutical proteins (Carter et al., 2002). The leaf biomass produced by alfalfa is somewhat lower than that of the model plant tobacco, but it has several advantageous agronomic characteristics compared to tobacco including the fact that it is a perennial plant (vegetative growth can be maintained for many years), it can be clonally propagated by stem cutting, and its leaves or free of alkaloids. Moreover, since alfalfa is a fodder crop, a major application of this species in molecular farming is the delivery of vaccines to domestic animals (Wigdorovitz et al., 1999). Alfalfa plants used in the present study showed high level of VP1 expression when transiently transformed with *A. tumefaciens*. This high level of gene expression was evident in both transcription (Figure 3) and translation levels (Figure 5). Although it is more reasonable to perform a stable genetic transformation program for permanent production of recombinant vaccine, transient gene expression can be regarded as a complementary process for achieving large amount of antibody for detection methods such as ELISA, western blotting, etc. A practical example of transient gene expression of antigens in alfalfa leaves is the case of Medicago Inc., Québec, Canada; the Scientists at this biotechnology company regularly process up to 7500 infiltrated alfalfa leaves per week for diagnostic objectives (Fischer and Schillberg, 2004).

The antigen can be quickly expressed in plant system through transient gene expression and the expressed recombinant protein can be parenteraly injected to animal models. This will trigger antibody production in immune system of the recipient animal. Based on

the results, alfalfa is an appropriate platform for production of recombinant antigen of FMDV. The transformed alfalfa lines can be parenteraly injected or orally administered to animals, because the crop is a palatable plant that can be easily incorporated in animal diet.

Conclusion

In this investigation, the efficacy of agroinfiltration for transient expression of VP1 protein in alfalfa plants was demonstrated. The expression level of the foreign gene was quite high in transformed plants (0.95% of TSP). We believe that this method can be used as an effective and quick way for the production of recombinant antigens. The expressed antigen can be used as a recombinant vaccine or, more realistically, as a valuable source for production of specific antibody in veterinary diagnosis or molecular detection processes

References

1. Bachrach H. L., Moore D. M., Mckercher P. D. Polatnick J. (1975) Immune and antibody response to an isolated capsid protein of foot and mouth disease virus. Journal of Immunology 115: 1636-1641
2. Bhat. R., Lahaye. T. and Panstruga. R. (2006) The visible touch: in planta visualization of protein-protein interactions by fluorophorebased methods. Plant Methods 2: 12-26.
3. Carter J. E. and Langridge W. H. R. (2002) Plant-based vaccines for protection against infectious and autoimmune diseases. Critical Reviews in Plant Sciences 21:93–109.
4. Doran P. M. (1999) Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 24:426− 432
5. Fischer R. and Schillberg S. (2004) Molecular Farming. WILEY-VCH Verlag GmbH & Co. KGaA,Weinheim. pp 329
6. Habibi M. and Zibaee S. (2013) Plant-based recombinant vaccines. International Journal of Agriculture and Crop Sciences 6: 27-30
7. Habibi-Pirkoohi M., Malekzadeh-Shafaroudi S., Marashi H., Moshtaghi N., Nasiri M. and Zibaee S. (2014) Transient expression of foot and mouth disease virus (FMDV) coat protein in Tobacco (Nicotiana tabacom) via Agroinfiltration. Iranian Journal of Biotechnology 12(3): 1015-1021
8. Ihara Y., Nagano M., Muto S., Uchimiya H. and Kawai M. (2007) Cell death suppressor Arabidopsis Bax inhibitor- 1 is associated with calmodulin binding and ion homeostasis. Plant Physiology 143: 650–660.
9. Janssen B. and Gardner R. (1989) Localized transient expression of GUS in leaf discs following cocultivation with Agrobacterium. Plant Molecular Biology 14:61–72.
10. Koprowski H. and Yusibov V. (2001) The green revolution: plants as heterologous expression vectors. Vaccine 19: 2735–41
11. Leckie B. M. and Stewart C. N. (2011) Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. Plant Cell Reports 30: 325–334.
12. Rodriguez L. L. and Grubman M. J. (2009) Foot and mouth disease virus vaccines. Vaccine 27 (4): 90-94.
13. Sala F., Rigano M., Barbante A., Basso B., Walmsley A. M. and Castiglione S. (2003) Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. Vaccine 21:803–808.
14. Santos-Rosa M. A., Poutaraud D., Merdinoglu D. and Mestre P. (2008) Development of a transient expression system in grapevine via agro-infiltration. Plant Cell Reports 27:1053-1063.
15. Sohn S., Huh S. M., Kim K. H., Park J. W. and Lomonossoff G. (2011) Effect of rice stripe virus NS3 on transient gene expression and transgene co-silencing. Plant Pathology Journal 27(4): 310-314.
16. Sparkes IA., Runions J., Kearns A. and Hawes C. (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nature Protocols 1(4): 2019-25.
17. Tang X., Frederick R. D., Zhou J., Halterman D. A., Jia Y. and Martin GB. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science 274: 2060–2063.
18. Vaquero C., Sack M., Chandler J., Jürgen

D., Schuster F., Monecke M., Schillberg S. and Fischer R. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. PNAS, Proceedings of the National Academy of Sciences 96:11128–11133.

19. Walmsley A. M. and Arntzen C. J. (2000) Plants for delivery of edible vaccines. Current Opinion in Biotechnology 11:126–9.
20. Wang C. Y., Chang T. Y., Walfield A. M., Ye J. and Shen M. (2002) Effective synthetic peptide vaccine for foot-and-mouth disease in swine. Vaccine 20: 2603–2610.
21. Wroblewski T., Tomczak A. and Michelmore R. (2005) Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis Plant Biotechnology Journal 3: 259–273.
22. Yasmin A., Jalbani A., Ali M., Nasreen A. and Debener T. (2013) Development of Agrobacterium-based transient gene expression assay in rose leaves. Pakistan Journal of Botany 45 (3): 1005-1009

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Research Article

Purification and Characterization of an Extracellular Phosphatase Enzyme From *Bacillus* spp.

Maryam Parhamfar[1]*, Arastoo Badoei-Dalfard[1], Milad Parhamfar[2] , Shohreh Fahimi Rad[3]

[1] *Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran*
[2] *Department of Chemistry, Faculty of Science, Duissburg-Essen University, Essen, Germany*
[3] *Department of Biotechnology, Campus of Agriculture and Natural Resources, University of Tehran, Karaj, Iran*

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Abstract

Phosphorus is one of the most important nutrients for plant growth and development. Chemical Pi fertilizer is used to provide the phosphorus for the plants, but it is mostly fixed in the soil into insoluble form and become unavailable to the plants. Phosphate-solubilizing bacteria have lots of application in agriculture as biological fertilizer. Consumption of biofertilizers instead of chemical fertilizers can lead to environmental pollution reduction and crop production enhancement using sustainable farming. In this study, a phosphatase-producing bacterium was isolated from agricultural soil in Kerman. Screening of phosphate solubilizing bacteria was performed on the PVK medium, based on clear area diameter. The best bacterium (AG41) was identified based on 16s rDNA gene. The optimum condition for production of phosphatase was also determined and it was purified and characterized. Sequence alignment and phylogenetic tree results show that AG41 is closely related to *Bacillus subtilis*, with 98% homology. Phosphatase activity was determined by end point method. The best carbon, nitrogen and phosphate sources for enzyme production were 1.0% glucose, 0.5% ammonium sulfate and (0.25%) sodium phytate +(0.25%) tricalcium phosphate, respectively. Bacterial phosphatase was partially purified using ammonium sulfate fractionation followed by dialysis. Results showed that the optimum temperature for the purified enzyme activity was 40ºC and it was stable at temperatures below 60°C. This enzyme was stable between pH 3.0-7.0, and the optimal pH activity was found to 5.0. These results indicated that this strain can be a notable candidate for using as biofertilizers.

***Keywords*:** Screening, Biofertilizer, Phosphate-solubilizing bacteria, Phosphatase

Introduction

Phosphorus (P) is one of the most important essential elements for crop production. Although phosphorus is quite abundant in many soils, it is one of the major nutrients limiting plant growth (Vassilev and Vassileva, 2003). With increasing demand of agricultural production and as the peak in global production will occur in the next decades, phosphorus is receiving more attention as a non renewable resource (Shen et al., 2011). Phosphorus is added to soil in the form of phosphate fertilizers but the overall P use efficiency is low because a large portion of the soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Rodríguez and Fraga, 1999). In addition, applications of chemical P fertilizers and animal manure to agricultural land cause environmental damage in the past decades (Shen et al., 2011).

On the other hand, several phosphate solubilizing bacteria (PSB) occur in soil, but usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for increase in situ plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Rodríguez and Fraga, 1999). Biofertilizers are the microorganisms that can convert useless nutrient to usable compounds. Using biofertilizers containing Pi solubilising bacteria hydrolyses P from various phosphate compounds resulting in decreasing application of the chemical Pi fertilizers. The principal mechanism of phosphate solubilization

Corresponding authors E-mail:
* *mary_parhamfar@yahoo.com*

bacteria for mineral phosphate solubilization is the production of organic acids and acid phosphatases (Rodríguez and Fraga, 1999). Actually, the major source of phosphatase activity in soil is considered to be of microbial origin (Sharma et al., 2013). Phosphatases or phosphoric monoester hydrolases (EC 3.1.3) are clustered in acid or alkaline. The acid phosphohydrolases, show optimal catalytic activity at acidic to neutral pH values. Also, they can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity (Rodríguez and Fraga, 1999). Secretion of phosphatase enzymes occurs in response to both phosphate starvation and environmental pH signaling, instigating the microbes to utilize phosphorus containing substrates (Ferreira-Nozawa et al., 2003). On the other hand, activity of phosphatase enzyme is affected by some factors, such as the amount and type of substrate, pH, temperature, concentration of enzyme and product (Fitriatin et al., 2011). It should be noted, proper and economical production of biofertilizers depends on the mass production of biofertilizer forming microorganisms which are obtained by suitable culture medium and fermentation process (Nautiyal et al., 2000).

In this study, phosphate solubilizing bacteria was isolated and characterized from an agricultural soil in tropical regions. Bacteria with the highest phosphatase producing ability purified on the specific media and identified. The optimum condition for growth and production of phosphatase was also determined. Phosphatase from bacteria was partially purified and stability and activity of phosphatase was assayed in different temperatures, pHs.

Materials and Methods

Screening of Phosphate-Solubilizing Bacteria (PSB)

Samples picked up from agricultural soil in Kerman, located the south-eastern of Iran. 1 g of soil samples was suspended in 10 ml of 0.9% saline solution and 1 ml of this suspension was inoculated in 50 ml of Pikovskaya (PVK) broth [1% glucose, 0.5% $Ca_3(PO_4)_2$, 0.05% Yeast extract, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% $MgSO_4.7H_2O$, 0.02% NaCl, 0.0002% $FeSO_4$, 0.0002% $MnSO_4$, pH 7] and were incubated for 2 days at 37ºC in an orbital shaker (180 rpm) (Shekhar Nautiyal, 1999). Then 0.1 ml of the cultured samples were streaked on PVK agar (at 37ºC for 48 h). A clearing zone around the bacterial colony on PVK plates represents extracellular phosphatase activity (Hu et al., 2010; Fitriatin et al., 2011). A promising colony showing the highest clear zone was purified on the specific media and designated as AG41. Isolate AG41 was selected for further study and identified up to the genus level based on the morphological and biochemical properties.

PCR Amplification and 16S rDNA Sequencing

Genomic DNA of isolate AG41 was extracted according to Sambrook and Russell protocols (Sambrook and Russell, 2001) and its purity was checked by the A260/A280. Then universal 16S rRNA PCR forward primer (5-AGTTTGATCCTGGCTCAG-3) and reverse primer (5-GGC/TACCTTGTTACGACTT-3) were used for the amplification of 16S rRNA gene. The reaction conditions were as follows: (1) 94°C for 5 min as initial temperature, (2) a run of 30 cycles with each cycle consisting of 45 s at 94°C, 45 s at 54°C and 90 s at 72°C, and (3) 8 min at 72°C to permit for the extension of any incomplete products. Products were purified by DNA extraction kit (Cinaclone) and DNA sequencing was performed on both strands directly by SEQ-LAB.

The phylogenetic tree was made based on the comparison of 16S rRNA sequences of *Bacillus* sp. AG41 strain with other strains of *Bacillus* species that were obtained from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov). All sequences were aligned with Clustal Omega that was obtained from: http://www.seqtool.sdsc.edu/ CGI/Omega.cgi (Sievers et al., 2011) and phylogenetic tree was made in the MEGA program version 4 (Tamura et al., 2007).

Optimization of Culture Media for Phosphatase Production

Effect of Carbon Source on Phosphatase Production

Effect of different carbon sources on phosphatase production was determined by addition of 0.1% of respective sugar (1% galactose and 0.5% glucose + 0.5% galactose) instead of glucose in the PVK medium. Strain AG41 incubated in this medium in shaker at 37°C for 3 days. Samples were picked up at each 24 h interval and phosphatase activity was determined. Phosphatase activity and phosphate releasing was determined by end point method.

In this method, 1 ml of medium containing grown bacteria was centrifuged for 10 min at 4000 rpm. The reaction mixture consisted of 100 µl supernatant (enzyme) and 300 µl 0.1 M sodium acetate buffer of pH 6.0, containing 2 mM sodium phytate (substrate)

and 2 mM $CaCl_2$. The reaction mixture was incubated at 37°C for 30 minutes then stopped by adding 400 µl of 5% (w/v) trichloroacetic acid (TCA). Then 400 µl of ammonium molybdate reagent was added (containing 1:4 mixture of 2.7% FeSO4 and 1.5% ammonium molybdate in 4.4% H_2SO_4).

After 10 min incubation at room temperature, absorbance at 700 nm was read. Measurement of phosphatase activity is based on the colorimetrical quantification at 700 nm of free phosphorus released by the hydrolysis of phosphate, using ammonium molybdate as color reagent (molybdate-blue method) (Kerovuo and Tynkkynen, 2000). One enzyme unit (U) is the amount of enzyme librating 1 µM of inorganic phosphate in 1 minute under the assay.

Effect of Nitrogen Source on Phosphatase Production

To determine the effect of nitrogen source on phosphatase production, the activity of this enzyme in PVK medium with 0.5% glycine, 0.5% nitrate sodium and 0.5% ammonium sulfate was assayed. The best strain (AG41) was inoculated into 50 ml of PVK broth in a 250 ml erlenmeyer flask and incubated on shaker a 37°C for 3 days.

Effect of Phosphate Source on Phosphatase Production

To study the effect of phosphate on phosphatase production, the activity of this enzyme in nutrient broth, PVK medium with tricalcium phosphate (0.5%), sodium phytate (0.5%) and medium with sodium phytate (0.25%) + tricalcium phosphate (0.25%) was assayed. The medium was incubated under same condition as above. The enzyme activity was measured for 3 days. The chosen carbon, nitrogen and phosphate sources were used in the subsequent experiments.

Partial Purification of Enzyme and Phosphatase Activity Assay

Partial purification of phosphatase was by ammonium sulfate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulfate up to 65%. The content was incubated over night and centrifuged at 12,000 rpm for 10 min. Supernatant was collected and investigated for enzyme activity. Pellet was collected and transferred to dialysis bag and immersed in Tris/HCl buffer (pH 7.5) at 4°C for 24 h. Then phosphatase activity was determined by end point method.

Effect of Temperature on Enzyme Activity and Stability

Phosphatase activity was determined at various temperatures as following; 10-70°C. For the thermostability, the enzyme was incubated for 30 min at various temperatures as described previously. Then phosphatase activity and phosphate releasing was determined by molybdate-blue method.

Effect of pH on Enzyme Activity and Stability

Phosphatase activity was determined at various pHs as following; pH 3-10. For the pH stability, the enzyme was incubated at various pH for 30 min as described previously. The residual activity was determined.

Results

Screening and Identification of Phosphatase Producing Bacteria

A total of 20 strains that can solubilize tricalcium phosphate and form peripheral halo zone on phosphatase specific agar medium around colonies were isolated. Amongst these, isolate AG41 showing the highest clear zones on PVK screening medium was selected for the further studies (Figure 1). Based on their cultural, biochemical and morphological characteristics, AG41 isolate was identified that belong to *Bacillus* genus.

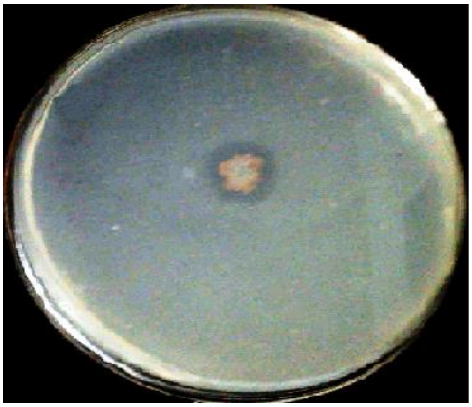

Figure 1. Zones of clearance by AG41 isolate on PVK screening medium

Bacillus isolate (AG41) was subjected to molecular identification using 16S rRNA. The PCR product of 16S rRNA gene was about 1400 bp. The phylogenetic tree was made by neighbor-joining method by MEGA4 software (Figure 2) (Sievers et al., 2011). Result shows that *Bacillus* sp. AG41is strongly related to *Bacillus subtilis* with 98% homology.

Research Article

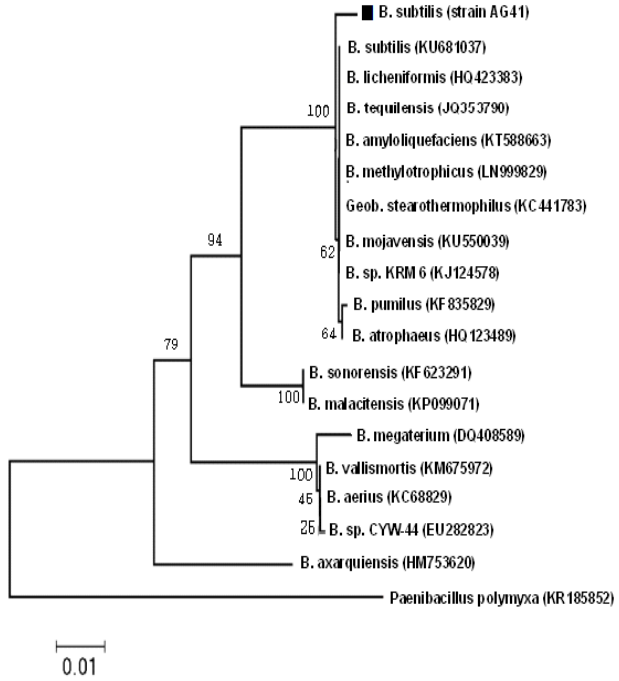

Figure 2. The phylogenetic tree constructed by the neighbor-joining method showing the position of isolate AG41

Optimization of Culture Condition

Among different carbon sources studied, glucose showed the maximum phosphatase activity (Figure 3).

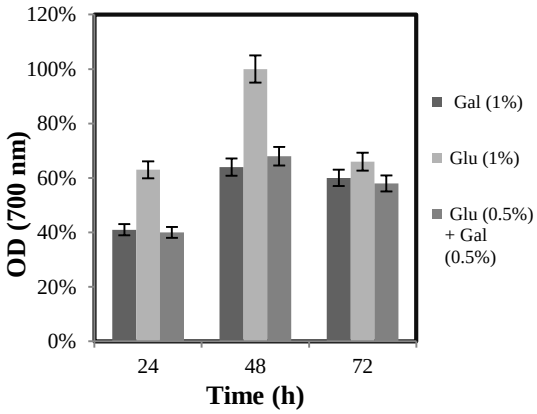

Figure 3. Effect of carbon source on phosphatase production

Results show that all three carbon sources improved phosphatase production at 48 h of incubation and slightly decrease at 72 h.

To investigate the effect of nitrogen sources on the growth and production of phosphatase, ammonium sulfate showed the maximum enzyme production and phosphate releasing (Figure 4).

Assay activity of this enzyme carried out in four media with different phosphorus sources.

Result showed phosphatase activity in medium with sodium phytate (0.25%) + tricalcium phosphate (0.25%) increased after 2 days.

Thus, this enzyme seems to enable degradationof sodium phytate through possibly phosphatase with phytase activity (Figure 5).

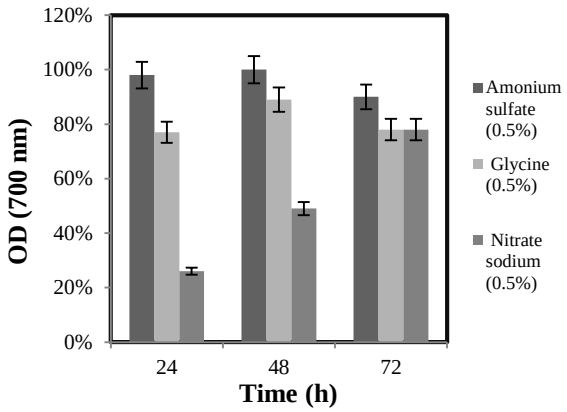

Figure 4. Effect of nitrogen source on phosphatase production

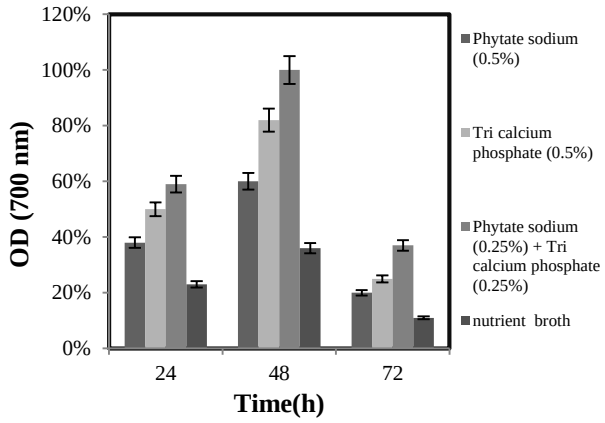

Figure 5. Effect of phosphate source on phosphatase production

Enzymatic Properties

Temperature Optimization and Thermal Stability

The temperature profile of the purified phosphatase was determined from 10°C to 70°C using the standard phosphatase assay. The optimum temperature was found to be 40°C (Figure 6a).

The apparent activation energy was estimated at pH 6.0.

In order to check thermal stability, the purified enzyme was incubated at different temperatures,

cooled to 4°C and assayed using the standard phosphatase assay.

The data showed excellent linearity from 10°C to 50°C. This phosphatase enzyme, when exposed for 30 min at 60°C, retained 80% and at 70°C, 50% of the initial activity, which indicate that the enzyme is thermostable (Figure 6b).

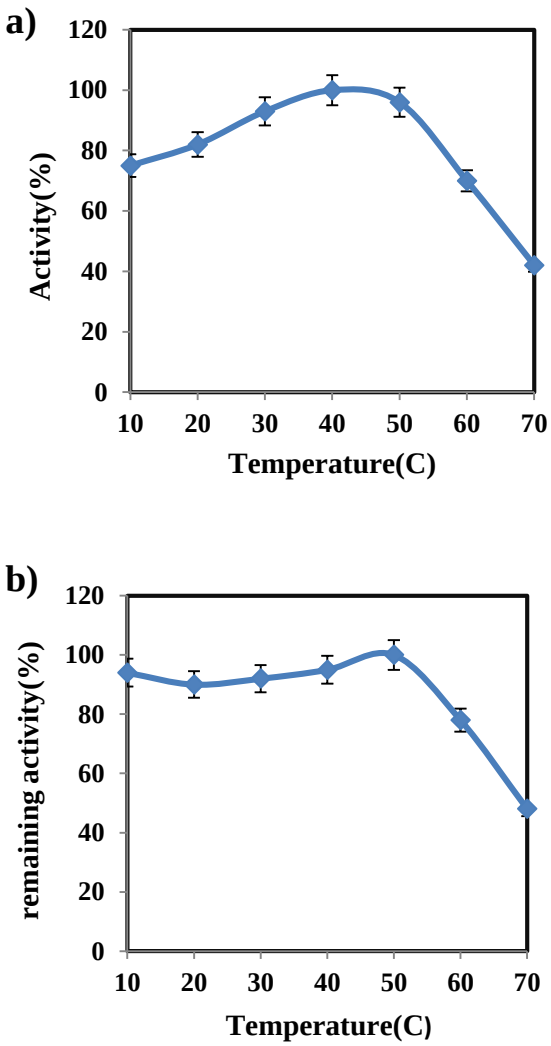

Figure 6. Effect of temperature on phosphatase activity (a) and stability (b) of *Bacillus* sp. AG41.

pH Optimization and pH Stability

The purified enzyme had a single optimum pH at pH 5.0. At pH 6.0, 82% and at pH 7.0, 76% of the activity at optimal pH was observed.

The effect on enzyme stability was studied in the pH range 3.0–10.0 at 4°C. Within 30 minutes the phosphatase did not show activity in the pH 10.0, but at pH 6.0, 98% and at pH 8.0, 75% of the initial activity was retained (Table 1).

Table 1. Effect of pH on phosphatase activity and stability

pH	Activity (%)	Remaining activity (%)
3	50	90
4	90	95
5	100	100
6	82	98
7	76	90
8	56	75
9	45	40
10	30	17

Discussion

Phosphatase is one of the important enzymes produced by several soil microorganisms. Also, the pH of most soils ranges from acidic to neutral values. Thus, acid phosphatases should play the major role in phosphate solubilization. (Rodríguez and Fraga, 1999).

On the other hand, there are fewer reports of phosphatase activity shown by microbes in tropical soils (Nopparat et al., 2007; Nenwani et al., 2010). Currently, most of PSB studied and applied to date have been mesophiles that could only be used under mesophilic conditions (Jatoth et al., 2015). Therefore, these types of microbes are not appropriate as biofertilizer for tropical regions. Also, they are not suitable for biofertilizer preparation at the high temperatures (over 50ºC) that occur during the first stage of composting (Chen et al., 2007). Thus, it is very important and necessary to isolate and screen some phosphate-solubilizing bacteria that can adapt to the environment for enhancing the utilization of phosphorus in the agriculture. Thermal stability of phosphatase is considered to be an important and useful criterion for application in agriculture as biofertilizers in acidic and tropical soils.

In this study soil sample of agriculture (in tropical regions) was chosen to isolate the phosphate solubilisers. The bacterial strain isolated from soil will have phosphate solubilizing capacity by producing extracellular phosphatase. 20 strains

showed phosphatase activity on PVK agar. The highest phosphatase producing strain, AG41, was selected based on a clear zone around the strain. In present study, strain AG41 is strongly related to *Bacillus subtilis* using 16S rDNA sequencing analysis.

The parameters like carbon, nitrogen and phosphate sources were optimized for better phosphate solubilization and production of phosphatase enzyme. The nature of the carbon compound and the concentration may stimulate or down modulate the production of enzymes in the microorganisms. Among the different carbon sources, 1.0% glucose shown the maximum production of enzyme. Nitrogen is considered as another energy source for growth of the microorganism and phosphatase production. The better nitrogen source for enzyme production and phosphate releasing was ammonium sulfate. Also, the enzyme production was maximum when the medium was modified with sodium phytate (0.25%) + tricalcium phosphate (0.25%) as phosphate sources.

The best carbon and phosphate sources for maximum phosphatase production by *Bacillus licheniformis* isolated from hot spring were glucose and tricalcium phosphate, respectively (Parhamfar et al., 2014). Phosphate solubilization activity of *Aspergillus* sp. was investigated in the presence of five carbon and seven nitrogen sources. This strain demonstrated diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources, but glucose and $(NH_4)_2SO_4$ were found as the best carbon and nitrogen sources (Pradhan and Sukla, 2012). It was previously reported that the best carbon and nitrogen sources for production of alkaline phosphatase by *Bacillus subtilis* were starch and Egg albumin, respectively (Jatoth et al., 2015).

Partial purification of enzyme phosphatase was by ammonium sulfate precipitation followed by dialysis. The optimum temperature for enzyme purified activity was 40ºC and the enzyme was stable at temperatures below 60°C when preincubated at various temperatures for 30 min. Cheng and Yang (2009) reported Thermo-tolerant phosphate-solubilizing microbes with six types of enzyme activities and three types of inorganic phosphate-solubilizing activities at 25 and 50ºC were isolated from the composts and biofertilizers. Acid phosphatase of *Penicillium citrinum* (cold-tolerant fungus) had an optimum temperature of 60ºC. The dried enzyme extract is stable at a temperature of up to 50ºC for at least 1 h (Gawas-Sakhalkar et al., 2012). In addition, pH had a statistical effect on the activity of acid phosphatase where, in more acid media, a higher activity of acid phosphatase was evaluated. As an example, Mclachlan (1980) reported the greatest phosphatase activity in the acidic range pH optima 5-6 for all species. It seems, increase in pH effect the charges on the amino acids with in the active site such that the enzyme is not to be able to form enzyme substrate complex. Therefore, there is decrease in enzyme activity (Mahesh et al., 2010).

The optimum pH of the AG41 phosphatase was 5.0 and it was relatively stable over a pH range of 3.0 to 7.0. So, it can be concluded that the purified enzyme phosphatase is a thermostable acid phosphatase with phytase activity. Acid phosphatase isolated from *Burkholderia gladioli* was stable after 6 h of incubation at 45°C in 100 mM acetate buffer at pH 6.0. The rate of hydrolysis enzyme reached a maximum at pH 6.0 (Rombola et al., 2014). Boyce and Walsh (2007) reported an acid phosphatase with activity on phytate produced by *Mucor hiemalis Wehmer* that exhibited a maximum activity at a temperature of 55ºC and pH 5.0-5.5. An alkaline phosphatase was produced from *Bacillus* spp, isolated from soil samples shows its optimum activity at pH 8.8 and temperature 65ºC, which indicate that the enzyme is thermostable (Mahesh et al., 2010).

This study reports biological production of phosphatase by *Bacillus* sp. AG41. Enzyme was able to tolerate high temperature and acidic pH. In addition, phosphatase enzyme production capacity, activity and stability in various conditions and phosphate solubilizing potential in different carbon and phosphate sources show that this strain has considerable importance as biofertilizers. Also, efforts have been made to encapsulate phosphate solubilizing bacteria for use in agriculture because these bacteria involved in both mineral and organic phosphate solubilization and increase soluble phosphorus in soil, stimulate root growth and promote sprouting on different plant species through the increase in P uptake.

References

1. Boyce A. and Walsh G. (2007) Purification and characterization of an acid phosphatase with phytase activity from *Mucor hiemalis Wehmer*. Journal of Biotechnology 132(1): 82-7.
2. Chang C. H. and Yang S. S. (2009) Thermo-tolerant phosphate-solubilizing microbes for multi-functional

biofertilizer preparation. Bioresource Technology 100: 1648–1658.

3. Chen K. S., Lin Y. S. and Yang S. S. (2007) Application of thermotolerant microorganisms for biofertilizer preparation. Journal of Microbiology, Immunology and Infection 40(6): 462-73.
4. Ferreira-Nozawa M. S., Nozawa S. R., Martinez-Rossi N. M. and Rossi A. (2003) The dermatophyte Trichophyton rubrum secretes an EDTA-sensitive alkaline phosphatase on high-phosphate medium. Brazilian Journal of Microbiology 34(2): 161-164.
5. Fitriatin B. N., Arief D. H., Simarmata T., Santosa D. A. and Joy B. (2011) Phosphatase-producing bacteria isolated from Sanggabuana forest and their capability to hydrolyze organic phosphate. Journal of Soil Science and Environmental Management 2(10): 299-303.
6. Gawas-Sakhalkar P., Singh S. M., Naik S. and Ravindra R. (2012) High-temperature optima phosphatases from the cold-tolerant Arctic fungus *Penicillium citrinum*. Polar Research.
7. Hu X. J., Li Z. J., Cao Y. C., Zhang J., Gong Y. X. and Yang Y. F. (2010) Isolation and identification of a phosphate-solubilizing bacterium *Pantoea stewartii* subsp. *stewartii* g6, and effects of temperature, salinity, and pH on its growth under indoor culture conditions. Aquaculture International 18(6): 1079–1091.
8. Jatoth K., Shantipriya A., Mangilal T. and Junapudi S. (2015) Optimization for the production of extracellular alkaline phosphatase from *Bacillus subtilis*. International Journal of Current Microbiology and Applied Sciences 4(10): 829-838.
9. Kerovuo J. and Tynkkynen S. (2000) Expression of *Bacillus subtilis* phytase in *Lactobacillus plantarum* 755. Letters in Applied Microbiology 30: 325-329.
10. Mahesh M., Neha G., Rajesh T. S., Somashekhar R. and Puttaiah E. T. (2010) Isolation and characterization of extracellular thermostable alkaline phosphatase enzyme from *Bacillus* spp. International Journal of Applied Biology and Pharmaceutical Technology 1(1): 21-33.
11. Mclachlan K. D. (1980) Acid phosphatase activity of intact roots and phosphorus nutrition in plants. I. Assay conditions and phosphatase activity. Australian Journal of Agricultural Research 31: 429-440.
12. Nautiyal C. S., Bhadauria S., Kumar P., Lal H., Mondal R. and Verma D. (2000) Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiology Letters 182(2): 291-6.
13. Nenwani V., Doshi P., Saha T. and Rajkumar S. (2010) Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity. Journal of Yeast and Fungal Research 1: 9-14.
14. Nopparat C., Jatupornpipat M. and Rittiboon A. (2007) Isolation of phosphate solubilizing fungi in soil from Kanchanaburi, Thailand. KMITL Science and Technology Journal 7: 137-146.
15. Parhamfar M., Badoei-Dalfard A., Khaleghi M. and Hassanshahian M. (2014) Isolation of phosphatase-producing phosphate solubilizing bacteria from Loriya hot spring: Investigation of phosphate solubilizing in the presence of different parameters. Biological Journal of Microorganism 3(9): 75-88.
16. Pradhan N. and Sukla L. B. (2005) Solubilization of inorganic phosphates by fungi isolated from agriculture soil. African Journal of Biotechnology 5(10): 850-854.
17. Rodríguez H. and Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17: 319–339.
18. Rombola T. H., Pedrinho E. A., de Macedo Lemos E. G., Gonçalves A. M., dos Santos L. F. and Pizauro J. M. (2014) Identification and enzymatic characterization of acid phosphatase from

Burkholderia gladioli. BMC Research Notes 7: 221.

19. Sambrook J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
20. Sharma S. B., Sayyed R. Z., Trivedi M. H. and Gobi T. A. (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus 2:587.
21. Shekhar Nautiyal C. (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiology Letters 170(1): 265-270.
22. Shen J., Yuan L., Zhang J., Li H., Bai Z., Chen X., Zhang W. and Zhang F. (2011) Phosphorus dynamics: From soil to plant. Plant Physiology 156: 997–1005.
23. Sievers F., Wilm A., Dineen D. G., Gibson T. J., Karplus K., Lopez W., Li R., McWilliam H., Remmert M., Söding J., Thompson J. D. and Higgins D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology 7: 1-9.
24. Tamura K., Dudley J., Nei M. and Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.
25. Vassilev N. and Vassileva M. (2003) Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. Applied Microbiology and Biotechnology 61: 435–440

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